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Modification of the Biological Properties of Potato Tuber Membranes by Ionizing Radiation

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General Introduction

Treating foods with ionizing radiation is a powerful tool against preventable food loss and food borne diseases. It can be used (a) to inhibit sprouting of vegetables; (b) to delay ripening of fruits; (c) to kill insect pests in fruits, grains or spices; (d) to reduce or eliminate food spoilage organisms; and (e) to reduce food poisoning bacteria on some meat and sea food products [1]. Throughout 1960s and 1970s research works into technological aspects of food irradiation and the wholesomeness of irradiated foods have been conducted under worldwide cooperation, and in 1980 Joint FAO/IAEA/WHO Expert Commities on the wholesomeness of Irradiated Foods (JECFI) concluded that irradiation of any commodity up to an average dose of 10 kGy neither presented any toxicological hazard, nor introduced any special nutritional or microbiological problems [2]. Recently, environmental protection has become an urgent problem on a global scale with increasing interest in radiation treatment as a safe replacement for methylen bromide fumigation.

Sprout inhibition of potato tubers is one of the important applications of ionizing radiation to foods because of the considerable shelf-life extension that can be effected with a low dose. In 1936, Metlitsky reported that vegetable sprouting could be inhibited with X-rays. In 1950s, when radiation facilities became available industrially, radiation effect on sprouting was intensively studied and many investigations have confirmed the effectiveness of low-dose irradiation in sprouting control under different treatment conditions in various areas of the world [3].

In Japan, National Food Irradiation Research Project which dealt with the irradiation of 7 items of foods including potato tuber was promoted from 1967 to 1981. Based on the results of the studies in the projects, the Government cleared potato irradiation in 1972, and potato irradiation plant was set up at Shihoro Agricultural Co-operative Association in 1973. Since then potatoes have been irradiated with an annual production of 10,000 to 15,000 tons [3)-5)].

The feasibility study on food irradiation has almost been completed and technological difficulties in the practical use of ionizing radiation for sprouting control have been overcome. However, the scientific understanding of food irradiation is not necessarily sufficient; there is no clear explanation of the mechanism of sprouting

inhibition by ionizing radiation[3], and this situation introduces the negative attitudes of consumers against the irradiated potatoes. It is worth trying to study the mechanisms of physiological changes in potato tubers caused by ionizing radiation, which will contribute the elucidation of the mechanism of sprouting inhibition.

While nuclear DNA has been known to be the major target of ionizing radiation[6], membrane systems are the possible target contributing to the development of radiation effect on cells[7),8)]. Generally biological membranes are assemblies of lipid and protein molecules held together by noncovalent interactions. The dynamic fluid structure of biological membrane as shown in fluid mosaic model, is responsible for their various functions: (1) a selectively permeable barrier of cells; (2) energy and material production and (3) transduction of information from the exterior environment to the inner of the cells.

It has been reported that irradiation of potato tubers brings about various metabolic changes[3),9)] and some of these changes are attributable to the changes of membrane systems. For example increase in respiratory rate [10] in irradiated potato tubers would be dependent on the changes in mitochondorial membranes, and increases in sugar content [11] during post irradiation periods were explained by the changes in the permeability of amyloplast membrane, which play a central role in starch sugar conversion [12]. Alteration of impedance parameter has been reported as a useful identifying method of irradiated potato tubers[13], which also postulate radiation-induced membrane changes in potato tubers.

Responses of biological membranes in plants to environmental stresses have recently become an interesting subject among plant physiologists. Changes in lipid composition and the activity of membrane-bound enzymes represented by ion-translocating ATPase are actively studied for clarification of the mechanisms of the adaptation of plants to environment [14),15)]. While radiation can be regarded as one of the unique means giving stress to plant material, studies on radiation effects on membrane systems have focused on the animal cells and microorganisms [7)8)16)] and only limited biological data are available for plant materials.

The aim of this study is to elucidate the biological properties of potato tuber membranes modified by ionizing radiation at dose levels for sprouting inhibition. Two

types of radiation, gamma-rays from cobalt-60 and electron beams generated by accelerator, are currently available for food irradiation. At the beginning of the course of this study, in Chapter I, the evaluation method of the dose of gamma-rays and electron beams are established. In chapter II, based on the dosimetry, the comparative effects of the two types of radiations are studied in model membrane system using hydroperoxide formation as an indicator. The results of these studies indicated that the effects of gamma-rays on model membrane systems are much larger than those of electron beams. The following chapters deal with the effects of gamma-rays on the membrane systems of potato tuber; the lipid composition and metabolism (chapter III), and physicochemical properties and activity of membrane bound-enzyme (chapter IV). The characteristic changes in membrane lipid composition of irradiated potato tubers are observed, which are closely correlated with the changes of membrane functions such as electrolyte permeability and H^+-pumping.

These observations will contribute to the understanding of the mechanisms of sprouting control by ionizing radiation in the field of food irradiation and also to develop stress biochemistry of plants.

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Chapter I

Dosimetry of gamma-rays and electron beams with film dosimeter

Ionizing radiations which can be used for the treatment of foods are gamma-rays from Co-60 and Cs-137 and accelerated electrons from a machine at an energy of 10 MeV or lower. While most of the commercial food irradiation are conducted with gamma-rays from Co-60, accelerated electrons are increasingly utilized for treating foods. Gamma-rays are electromagnetic radiations which are converted into fast electrons in the medium through which they pass by Compton scattering, photoelectric absorption and pair-production. The reactions in the foods irradiated with gamma-rays are brought about mainly by the fast electrons thus formed, so the reactions caused by gamma-rays and those by electron beams from an accelerator are essentially the same. Whether or not there is any difference in the food and living organisms between gamma-rays and electron beams is controversial, although a lot of date are available on the comparative effects of the two types of radiation [1),2)].

The evaluation of radiation dose (dosimetry) is foundation of the comparative studies on the biological and chemical effects of gamma-rays and electron beams. Two kinds of dosimeters, cellulose triacetate dosimeter (CTA) and radiochromic film (RCF) have been used in the studies on the comparative bactericidal effectiveness. Studies in which doses were evaluated with CTA reported that the bactericidal effect of gamma-rays was higher than that of electron beams [3)-5)], and a study in which dose was evaluated with RCF reported that the effect of electron beams on dry preparation of bacterial spores was rather higher than that of gamma-rays [6)]. These report suggested that the inconsistency of comparative bactericidal effect of gamma-rays and electron beams among the studies is partly attributable to the difference in the dose measurement method employed. In this chapter, prior to the studies on the radiation effect on membrane systems, the response of RCF to gamma-rays and electron beams were compared with those of CTA to the two types of radiation by using the Fricke dosimeter as a standard and dose measurement procedure was established.

Materials and Methods

Film dosimeters

CTA (FTR-125, Fuji Photo Film Co.,Ltd.) and RCF (FWT-60-00, Far West Technology Inc.) were used in this study.

Irradiation of the film dosimeters

Unless otherwise stated, all the dosimeters were irradiated at National Food Research Institute (NFRI). Several pieces of CTA and RCF dosimeters were stacked together in layers and irradiated in a Gamma-cell 220 (2.1×10^{2}TBq of ^{60}Co, 4.6×10^{3}Gy/h, AECL) or in a Van de Graaff electron accelerator (2.5MeV, 147 μA, 1.5×10^{6}Gy/h, Nissin-High Voltage Co.,Ltd.), both of which were located at NFRI and operated at a temperature of 20-25°C and a relative humidity (R.H.) of 40-50%. The distance between the dosimeters and the window of the electron accelerator was 56 cm and the conveyer speed was 3 m/min. The film dosimeters were irradiated on a glass plate with a thickness of 1 cm, in order to irradiate the film dosimeters under conditions equivalent to those for Fricke dosimeter.

The film dosimeters were also irradiated at a temperature of 19°C and a R.H. of 69% in an electron linear accelerator (10MeV, 30μA, 2.2×10^{6}Gy/h, High Voltage Engineering Ltd.) and a water pool type gamma irradiation facility (4.4×10^{2} TBq of ^{60}Co, 9.0×10^{3} Gy/h) both of which were located at Research Institute for Advanced Science and Technology, University of Osaka Prefecture (RIAST).

Fricke dosimetry of gamma-rays and electron beams

Fricke solution (392 mg of $FeSO_4(NH_4)SO_4.6H_2O$ and 60 mg of NaCl in 1000 ml of 0.8N H_2SO_4) sealed with air in a glass ampoule was irradiated for different periods in the gamma-cell, and then its absorbance at 305nm ($O.D._{305}$) was measured against unirradiated Fricke solution. The same solution (5ml) was put in a glass petri dish with a diameter of 90 mm and irradiated with the electron accelerator in different beam currents and passes at a conveyer speed of 3 m/min, followed by the measurement of the $O.D._{305}$. The depth of Fricke solution in the petri dish was smaller than 0.8 mm and it was larger than the thickness of the irradiated film dosimeters. However, the difference of the dose build-up in the both dosimeters was estimated at a few percent or less. The absorbed dose in the Fricke solution was calculated as follows ;

$$\text{Dose(Gy)} = (2.75\times10^2 \times \text{O.D.}_{305}) / \{1+0.007 \times (t-25)\},$$

where t is the temperature (°C) of the solution.

Results and Discussion

Fricke dosimetry of gamma-rays and electron beams

The dose rate of gamma cell and dose per pass of electron beams were determined by Fricke dosimetry. The dose rate of gamma cell was about 1 kGy/13min (4.6×10^3Gy/h). The doses per pass of electron beams was shown in Table I-1.

Table I-1.
The doses per pass in electron beams irradiation with 2.5MeV at a conveyer speed of 3m/min.

beam current	dose per pass
3.0 μA	20 Gy
3.8 μA	26 Gy
5.9 μA	40 Gy
7.4 μA	50 Gy
7.6 μA	52 Gy
9.5 μA	65 Gy

The dose rates at which the fricke solution was irradiated with 2.5 MeV of electron beams were in a range of 3.0×10^4 to 1.0×10^5Gy/hr. They give the same G-value as 4.6×10^3Gy/h of Co-60 gamma-rays, because the G-value is constant up to the dose rate of 1.4×10^5Gy/h irrespective of type of radiation[7)-9)]. With the assumption that the dose of electron beams is directly proportional to beam current and conveyer pass, the dose per pass in electron irradiation at 147μA was about 1.0 kGy and the dose per pass at 190μA was about 1.3 kGy. These results indicate that 13.0 min gamma irradiation and single pass electron irradiation at 147μA gave the same dose of 1kGy.

Relationship between the change in absorbance at 280nm ($O.D._{280}$) of CTA and the change in absorbance at 510nm ($O.D._{510}$) of RCF at different dose rates

Several pieces of CTA and RCF were stacked together in layers and irradiated in the gamma-cell or in the Van de Graaff electron accelerator, and $O.D._{280}$ of CTA and $O.D._{510}$ of RCF were measured before and 2 h after irradiation to determine $O.D._{280}$ and $O.D._{510}$, respectively. The dose rate of electron beams was controlled by changing the distance between the accelerator window and the dosimeters. The curve obtained by plotting $O.D._{280}$ of CTA and $O.D._{510}$ of RCF for the gamma-cell was coincident with that for the electron accelerator, irrespective of the dose rate (Fig.I-1).

When the film dosimeters were irradiated in the irradiation reported to be dependent upon dose rate and type of radiation[10)-12)]. However, the results in this study demonstrated that the independence of RCF of dose rate and the dependence of CTA on dose rate were contradictory, as follows. Plotting $O.D._{280}$ of CTA and $O.D._{510}$ of RCF resulted in the same curve for gamma-rays and electron beams, when the dosimeters were simultaneously irradiated, as shown in Figs.I-1 and I-2, which indicates that CTA and RCF responded to the two types of radiations in the same manner. If the change in $O.D._{280}$ of CTA had been dependent upon type of radiation, the change in $O.D._{510}$ of RCF should have been also dependent upon type of radiation, and if the change in $O.D._{510}$ had been independent of type of radiation, the change in $O.D._{280}$ should have been also independent of type of radiation.

The changes in $O.D._{280}$ of CTA and $O.D._{510}$ of RCF by gamma-irradiation and electron-irradiation

CTA and RCF were irradiated for 130, 260 and 390 min in the gamma cell and irradiated at 147 μA or 190 μA at 10, 20 and 30 passes in the electron accelerator. The results are shown in Tables I-2 and I-3.

Table I-2. The changes of O.D.s of CTA and RCF in gamma-cell

Irradiation time	Dose	OD_{280} of CTA	OD_{510} of RCF
130 min	10 kGy	0.082±0.001	0.097±0.002
260 min	20 kGy	0.169±0.003	0.193±0.004
390 min	30 kGy	0.243±0.003	0.287±0.004

Table I-3. The changes of ODs of CTA and RCF in the electron accelerator.

Beam current	Pass	Dose	OD_{280}	OD_{510}
147 μA	10	10 kGy	0.063±0.001	0.075±0.003
147 μA	20	20 kGy	0.125±0.003	0.157±0.003
147 μA	30	30 kGy	0.190±0.004	0.221±0.005
190 μA	10	13 kGy	0.082±0.002	0.098±0.003
190 μA	20	26 kGy	0.165±0.002	0.194±0.004
190 μA	30	39 kGy	0.246±0.001	0.289±0.004

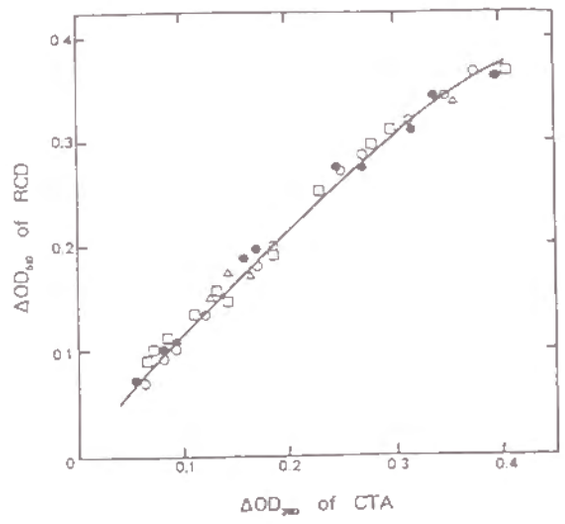

Fig. I-1. Relationship between $\Delta O.D._{280}$ of CTA and $\Delta O.D._{510}$ of RCF for the Gamma-cell 220 and the Van de Graff Electron Accelerator.

O : Electron accelerator, 1.5 x 10^6 Gy/hr

□ : Electron accelerator, 3.0 x 10^6 Gy/hr

Δ : Electron accelerator, 1.0 x 10^7 Gy/hr

● : Gamma-cell, 4.6 x 10^3 Gy/hr

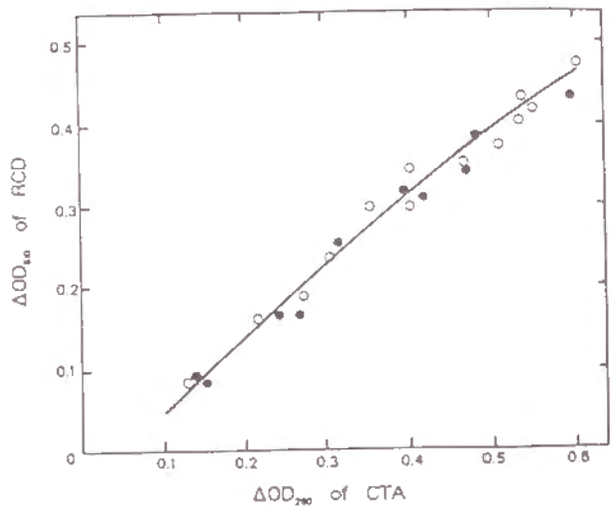

Fig. I-2. Relationship between $\Delta O.D._{280}$ of CTA and $\Delta O.D._{510}$ of RCF for the Water Pool Type Gamma Irradiation Facility and the Electron Linear Accellerlator.

O : Electron accelerator. 2.2 x 10^6 Gy/hr

● : Gamma irradiator. 9.0 x 10^3 Gy/hr

The changes in $O.D._{280}$ of CTA and $O.D._{510}$ of RCF by gamma-rays were different from those by electron beams, when the dosimeters were irradiated to the same dose. Both the changes in $O.D._{280}$ of CTA and $O.D._{510}$ of RCF by gamma-rays were same as those by electron beams, when the dosimeters were irradiated with electron beams to doses 30% higher than gamma-rays under low R.H. conditions such as 40-50%. These results indicate that both $O.D._{280}$ of CTA and $O.D._{510}$ of RCF are dependent upon dose rate under the irradiation conditions in this study. Because the responses of CTA and RCF to radiation are influenced by relative humidity and temperature during irradiation[12)-15)], it is necessary to compare the responses of CTA and those of RCF under various irradiation conditions to further clarify the dose rate dependence of CTA and RCF.

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Chapter II

Comparative effects of gamma–rays and electron beams on peroxide formation in phosphatidylcholine

Lipids have been known to be sensitive to ionizing radiation[1]. Mead et al.[2] reported the oxidation of linoleic acid in borate buffer. Petkau and Chelack[3] reported the conjugated dien formation in soy bean lecithin liposomes by gamma–irradiation. These reports indicated the decrease in lipid oxidation with the increase of dose rate of radiation within a dose rate range up to 50 Gy/min.

The dose rates of electron beams are 10^3 to 10^6 times higher than those of gamma–rays. Tuab et al.[4] and Azuma et al.[5] reported that the chemical effects of gamma–rays were higher than those of electron beams. Diehl [6]–[8] did not observe any dose rate effect and difference in the effect between gamma–rays and electron beams on thiamine and tochopherol. Thus whether the effect of gamma–rays and those of electron beams are same or not and whether there is any dose rate effect in the reactions caused by irradiation or not have been contraversal problem.

This chapter dealt with the comparative effectiveness of gamma–rays and those of electron beams on peroxidation of phosphatidylcholine, one of the major lipids composing biomembranes.

Materials and Methods

Preparation of film and liposome of phosphatidylcholine

Ten μl of 20mg/ml L–a–phosphatidylcholine(PC) (from egg, Avanti Inc.) in chloroform was dried under a nitrogen stream in a quartz cup to prepare film of PC.

Multilamellar liposome was prepared according to Lim et al.[9] and Verma and Rastogi[10] with a slight modification, as follows. Five mg of PC and 1 ml of 0.9% NaCl were mixed to prepare a suspension of PC. Multilamellar liposome of PC was obtained by agitating the suspension for 1 min with a vortex mixer followed by ultrasonic irradiation for 30 s in a Branson sonifier.

Bacteriorhodopsin (from Halobacterium halobium, Sigma Chemical Company) and ATPase (from dog kidney, Sigma Chemical Company) were used to prepare

proteoliposome. Proteoliposome of PC and protein was prepared in the same manner as liposome of PC except the addition of 1.0 ml of 1 mg/ml protein solution instead of 1 ml of 0.9% NaCl.

Irradiation of film and liposome of PC

A quartz cup with 200 μg of the PC film and a plastic petri dish with 100 μl of the liposome were packed in plastic bags, which were irradiated with a Gamma-cell 220 (5.9 kGy/h, AECL) and a van de Graaff electron accelerator (2.5MV, $1.5x10^3$Gy/h, Nissin High Voltage Engineering Co.). The dose rate of the Gamma-cell and the dose/pass of the electron irradiator were determined by Fricke dosimetry according to the method described in Chapter I; the dose rate was 1.0 kGy/13min, and one single pass of 147μA of 2.5 MeV electrons at a conveyer speed of 3 m/min gave 1.0 kGy. The dose absorbed by the sample was confirmed with radiochromic film dosimeter (FWT-60-00, Far West Technology Ltd.) which were attached to the surface of the plastic bag.

Determination of peroxide

Peroxide contents of PC film and liposome were determined 2 h after irradiation, according to Ohishi et al.[11] with a slight modification, as follows. Fifty μl of the liposome suspension was mixed with 0.9 ml of water, 1.0 ml of chloroform and 1.0 ml of methanol, and a chloroform layer was collected and dried under a nitrogen stream. The lipid fraction thus obtained and the PC film in the quartz cup were dissolved in 100 μl of isopropanol and the peroxide amount was determined with Determiner LPO kit (Kyowa Medics Co., Ltd.).

Results and Discussion

Peroxide amount of irradiated PC film

Irradiation with gamma-rays formed peroxides in the film of PC to a significantly greater degree than irradiation with electron beams ($p<0.01$)(Fig. II-1). No peroxide formation was observed in the PC film irradiated under an anaerobic condition with N_2 gas.

Peroxide amount in irradiated liposomes

The peroxide amount of gamma-irradiated liposome composed of PC was

higher than that of electron-irradiated one and the ratio of the peroxide amount of gamma-irradiated liposome to that of electron-irradiated one was 3 to 4, irrespective of radiation dose (Fig.II-2).

The peroxide amount of gamma-irradiated liposome composed of PC and bacteriorhodopsin (Fig.II-3) and that of PC and ATPase(Fig.II-4) were higher than that of electron-irradiated one. The ratio of peroxide amount of gamma-irradiated liposome to that of electron-irradiated one was 2 to 3 (Fig.II-3), although the peroxide amount was lower than that of liposome composed of only PC (Figs.II-2). These results indicate that the effect of gamma-rays on peroxide formation in PC are significantly larger than that of electron beams, irrespective of the state of lipid ($p<0.01$). The presence of proteins in liposomes inhibited peroxidation of lipid, which agreed with those reported by Verma and Rastogi[10] ; radicals formed by irradiation reacted with SH groups of proteins, which resulted in the reduced peroxidation of lipid.

As described in chapter I, film dosimeters such as radiochromic film dosimeter and cellulose triacetate film dosimeter were responded to gamma-rays to 30% greater degrees than electron beams. Hayashi et al.[12] also reported that the effects of gamma-rays on B.pumilus spores were about 30% higher than those of electron beams under aerobic conditions. The difference in the effect on peroxidation between gamma-rays and electron beams was much larger than those of previous observations. The difference in chemical and biological effects between the two types of radiation has been ascribed to both anoxic conditions and recombinations of radicals brought about by extremely high dose rate of electron beams[13]. Oxygen played a much more important role in the peroxidation of irradiated PC than in the color changes of film dosimeters(Chapter I) and the inactivation of B.pumilus spores[12]. With electron beams at high dose rates, oxygen in the reaction system was consumed for the peroxidation at a rate greater than it can be replaced by diffusion process transferring atmospheric oxygen into the system, which resulted in the decrease in the peroxidation rate of PC under anoxic conditions caused by electron irradiation. It is concluded that the effects of electron beams are smaller than those of gamma-rays and the difference of the effect between the two types of radiation is significantly large in the reactions in which oxygen plays an important role.

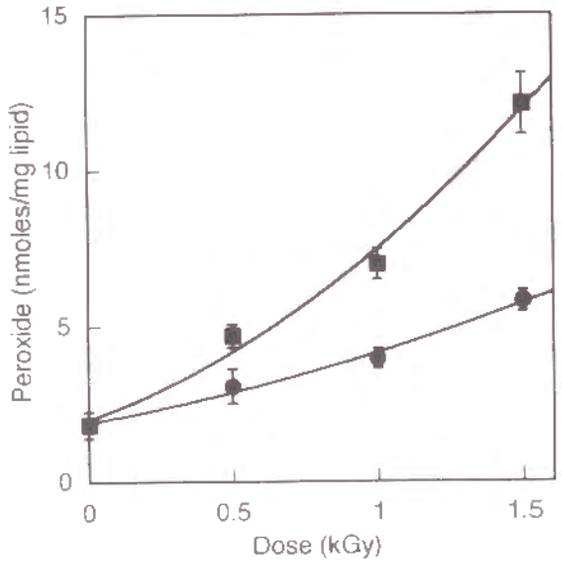

Fig. II - 1 Peroxide Amount in Irradiated PC Film.

● ; gamma-rays, ■ ; electron beams

Data are mean values and standard deviations of triplicate measurements.

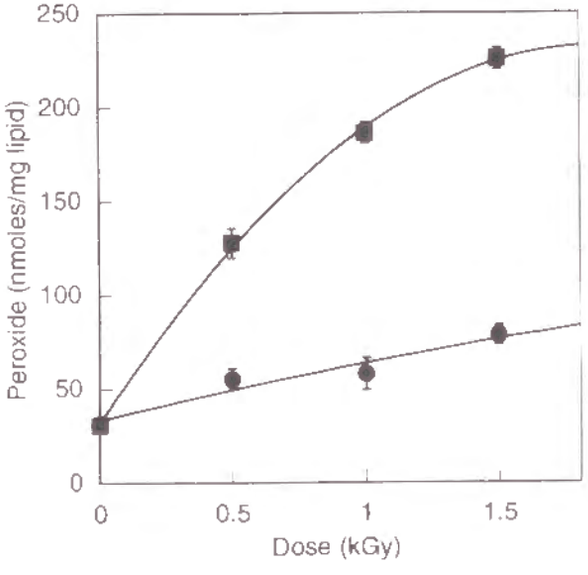

Fig. II - 2 Peroxide Amount in Irradiated Liposome Composed of PC.

● ; gamma-rays, ■ ; electron beams

Data are mean values and standard deviations of triplicate measurement.

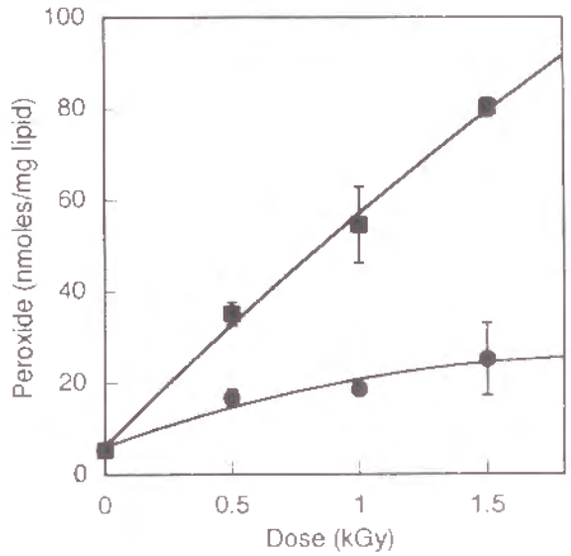

Fig. II - 3 Peroxide Amount in Irradiated Liposome Composed of PC and Bacteriorhodopsin. (PC : BR = 5 : 1 (W/W))

● ; gamma-rays, ■ ; electron beams

Data are mean values and standard deviations of triplicate measurements.

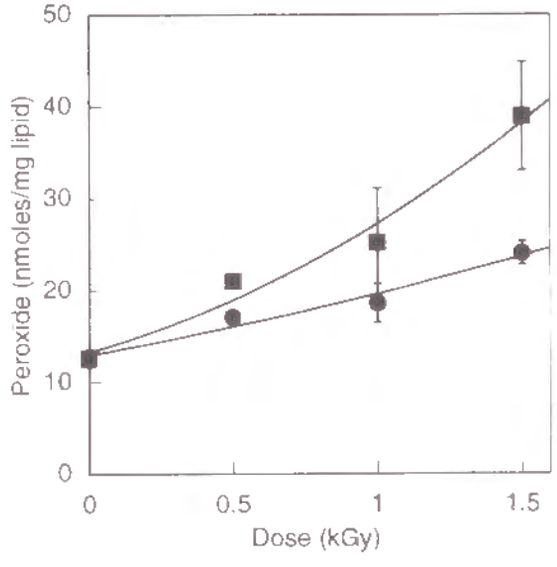

Fig. II - 4 Peroxide Amount in Irradiated Liposome Composed of PC and

$(K^+$-Na) ATPase. (PC : ATPase = 5 : 1 (W/W))

● ; gamma-rays, ■ ; electron beams

Data are mean values and standard deviations of triplicate measurements.

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Chapter III

Compositional Changes in lipids of gamma–irradiated potato tubers

Changes in membrane lipid composition have been observed in plants exposed to cold stress and correlated with adaptive properties of plants to low temperature.[1] For example, resistance of higher plants to chilling has been related to the fatty acid composition of phosphatidylglycerol of chloroplast membranes[2]. Storage of potato tubers at a low temperature (3 °C) caused an increase in membrane fatty acid unsaturatuion, which was inversely correlated with the increase in membrane electrolyte leakage and the accumulation of sugars[3].

It has been reported that mechanical injury causes membrane lipid breakdown in potato tubers, followed by membrane lipid synthesis[4]. This reconstitution of biological membranes has been considered to be essential for the development of wound respiration[5]. These reports[1)–5)] indicate the physiological importance of membrane lipids in stressed plants.

Irradiation of potato tubers has been reported to bring about the increase in respiratory rate[6] and sugar accumulation[7] as well as cold storage and mechanical stress. Therefore it is postulated that gamma–irradiation brings about the compositional changes of membrane lipids.

As shown in chapter II the effect of gamma–rays on lipids in model membrane system was much greater than that of electron beams, and this chapter dealt with the compositional changes of potato tuber lipids by gamma–irradiation.

III–1 Free fatty acid and lipid hydroperoxide of gamma–irradiated potato tubers.

As shown in Chapter II lipids in model membrane system was sensitive to radiation. At the beginning of this chapter, the effect of gamma–irradiation of potato tuber on the lipid hydroperoxide and free fatty acid(FFA) contents was examined within a short period after irradiation.

Materials and methods

Potato tuber

Potatoes (Solanum tuberosum cv. Danshaku) grown in Hokkaido were used in this study. Prior to irradiation, potato tubers were stored for one month at 5°C after harvest.

Irradiation of potatoes

Potatoes were irradiated at 0.5 kGy in a Gamma-cell 220 (AECL, 6.2kGy/h) unless otherwise stated. After irradiation potato tubers were stored at 5°C in a dark room.

Extraction and fractionation of lipids

All the extraction and fractionation procedures of lipids were carried out under an atmosphere of nitrogen and dim light.

Lipids were extracted from a potato tuber according to Bligh and Dyer[8]. Fifty g of potato tuber were incubated in 100ml of boiling iso-propanol and then homogenized with 150ml of chloroform:methanol (1:2), followed by filtration. The residue was homogenized with 190ml of chloroform:methanol:water (5:10:4, v/v) and filtered, followed by washing with 75ml of chloroform:methanol (1:2, v/v). All the filtrates collected were shaken with 125ml of chloroform and 145ml of water. The chloroform phase was evaporated with the iso-propanol fraction to dryness. The dried material thus obtained was dissolved in 125ml of chloroform, followed by washing with 240ml of methanol:water (10:9, v/v). The chloroform phase was evaporated to dryness. Total lipids thus obtained were dissolved in chloroform and stored at -80°C under N_2 condition.

Total Fatty acid composition

Transesterification of fatty acids was carried out by incubating the lipids in anhydrous methanol containing 5% HCl for 3h at 90°C. Fatty acid methyl esters thus obtained were analyzed with a Shimadzu GC-14A gas chromatograph (Shimadzu Seisakusho Ltd.) equipped with a flame ionizing detector and 0.25mm x 30m capillary column (DB-225 J&W Inc ltd.) The chromatograph was run isothermally at 190°C and detector and injector temperatures were 235 ° C.

Determination of FFA

Free fatty acid contents were determined by the analysis of 9-anthrldiasometane (ADAM, Funakoshi Co. Tokyo Japan) derivative of free fatty acid with a high performance liquid chromathograph according to the method of Nimura and Kinoshita [9] with slight modification. Twenty µl of lipid chloroform solution (containing ca. 1 nmole of FFA) were taken into glass vial and remove the solvent under N^2. The lipid was dissolved in 50 µl of 0.05% (w/v) ADAM methanol solution and for 1h for at 25°C, The ADAM derivative of FFA thus obtained was subjected to the HPLC analysis under the following conditions; column, NOVA PACK C18 (Waters) 3.9mm x 150mm; mobile phase, Acetonitrile / H_2O = 100/5 (1.0 ml/min); injection volume, 5 µl; detector, fluorescence detector (excitation 365 nm, emission 412nm).

Determination of lipid hydroperoxide

The contents of lipid hydroperoxide were determined with Determiner LPO kit as described in Chapter II.

Results and discussion

Figure III-1 shows the lipid hedroperoxide contents of potato tuber irradiated at up to 4 kGy and stored for 4h, and hydroperoxide contents of the irradiated lipids from potato tubers. The irradiation of lipids inereased the amount of hydroperoxide depending on radiation dose, however there was no clear relationship hetween the hydroperoxide contents and radiation dose in irradiation of potato tuber.

Table III-1 shows the contents of the sum of esterified and unesterified fatty acids (TFA), unesterifed free fatty acids(FFA) and lipid hydroperoxide in potato tubers during the storage after irradiation at 0.5 kGy. At the same time fatty acid composition of TFA and FFA were shown in table III-2. The increase in hydroperoxide was not observed during the storage after irradiation (Table III-1), which supported by the result of fatty acid composition in total lipid (TFA) (Table III-2). The percentages of poly-unsaturated fatty acid, linoleic acid (18:2) and linolenic (18:3), were not deceased by irradiation and storage. The percentages of 18:3 increased 10 days after irradiation as compared with the other ones.

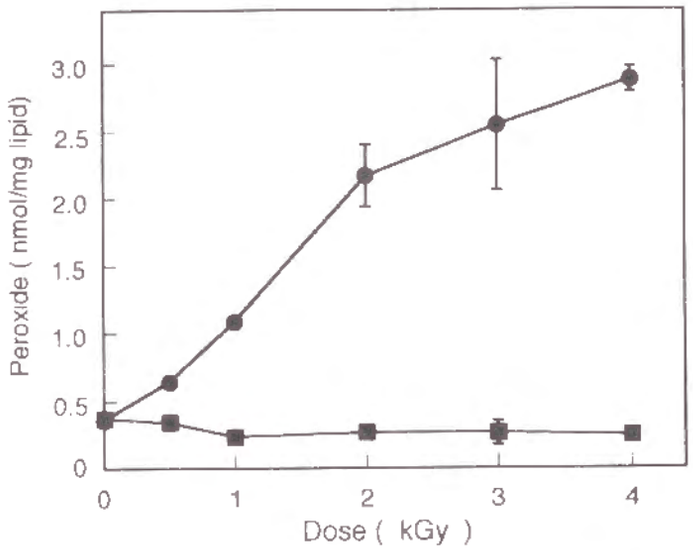

Fig. III - 1 Hydroperoxide Content of Irradiated Potato Tuber (■) and Irradiated Potato Lipid (●).
Results were shown as the mean value of three different analysis.

Table III-1 Changes in TFA , FFA and lipid hydroperoxide contents in gamma-irradiated potato tubers during storage at 5°C. (mean ± S.D. n = 3)

Dose (storage period)	TFA (μmol)	FFA (μmol)	FFA/TFA (%)	hydroperoxide (μmol)
unirradiated	242 ± 14	1.06 ± 0.01	0.44 ± 0.03	26.3 ± 1.7
0.5 kGy (0h)	249 ± 9	1.09 ± 0.03	0.44 ± 0.03	27.9 ± 1.7
0.5 kGy (12h)	235 ± 8	1.16 ± 0.01	0.49 ± 0.01	24.5 ± 0.3
0.5 kGy (24h)	245 ± 3	1.13 ± 0.05	0.47 ± 0.03	27.0 ± 0.1
0.5 kGy (10d)	244 ± 7	1.10 ± 0.1	0.45 ± 0.03	28.7 ± 0.9

Table III-2 Fatty acid composition of TFA and FFA

Dose	TFA composition				FFA composition				
(storage period)	16:0	18:0	18:2	18:3	16:0	18:0	18:1	18:2	18:3
unirradiated	21.1	3.4	56.2	16.2	38.8	12.0	8.2	29.5	11.5
0.5 kGy (0h)	22.4	3.2	56.3	15.6	38.0	12.5	8.0	30 0	11.5
0.5 kGy (12h)	21.8	3.4	55.4	16.7	39.9	11.0	8.1	28.5	12.0
0.5 kGy (24h)	21.2	3.4	55.6	16.6	34.7	10.0	6.4	34.0	14.5
0.5 kGy (10d)	20.4	3.5	50.2	22 9	35.2	10.5	6.3	31.0	17.5

Mean values of 3 different analysis.

It has been reported that *in vivo* irradiation of cauliflower bud did not brought about the increase in TBA value up to 4 kGy [10] and the hydroperoxide formation in irradiated erythrocyte membrane reported to be observed at doses over 0.5 kGy [11]. These results indicate that polyunsaturated fatty acids in biological membranes are stable as compared with model membrane systems because of the existence of radical scavenge system such as superoxide dismutase [12] and small anti-oxidant molecules such as tocopheroles[13].

Irradiation of potato tuber did not bring about significant decrease of TFA and increase of FFA ($p>0.05$) although the ratio of FFA to TFA (FFA/TFA) at 12h after irradiation slightly increased. Voisine et al.[10] reported that in vivo irradiation of cauliflower stimulated the senescence like membrane deterioration through lipolytic enzyme and brought about the increase in FFA level in its microsomal membrane

during the storage. In the present study, higher percentages of saturated fatty acid (16:0 and 18:0) were contained in FFA fraction as compared with TFA fraction even after irradiation, which suggest the remaining of acylhydrorase and lipoxygenase activities after irradiation of potato tuber. And the possibility of membrane degradation through these enzymes in irradiated tubers was not negligible because the ratio of FFA to TFA FFA/TFA slightly increased 12h after irradiation, however, in contrast to the irradiated cauliflower bud accumulation of FFA did not proceed during the storage after irradiation.

In conclusion irradiation of potato tuber did not brought about significant increase of lipid hydroperoxide nor FFA in its membrane lipids.

III-2 Changes in lipids of irradiated potato tuber during storage.

In the previous study the short term effects of gamma-irradiation on membrane lipid was examined. There was no clear evidence for membrane lipid degradation however the percentage of 18:3 increased 10 d after irradiation. The increased fatty acid unsaturation has been reported in potato tuber in response to the other stresses such as cold storage[3] and mechanical injury [4]. In this study in order to confirm the increase in 18:3, the lipid composition and content in irradiated potato tuber were investigated for a long term after irradiation.

Materials and Methods

Potato tubers

Potatoes (Solanum tuberosum cv. Dejima) grown in Nagasaki were used in this study. Prior to irradiation, potato tubers were stored for one month at 5°C after harvest.

Irradiation of potatoes

Potatoes were irradiated at 1kGy in a Gamm-cell 220 (AECL, 6.2kGy/h) unless otherwise stated. After irradiation potato tubers were stored at 5°C in a dark room.

Extraction and fractionation of lipids

Lipids were extracted according to the method previously described (Chapter III-1). The total lipids (ca. 50mg) were applied on a silic acid column (8g of Wakogel C-200, Wako Pure Chemical Industries Ltd.) and fractionated. The fractions eluted from the column with chloroform (90ml), acetone (350ml) and methanol (90ml) were labeled as neutral lipid, glycolipid and phospholipid fractions, respectively. The glycolipid and phospholipid fractions were further fractionated by thin layer chromatography on a Uni Plate (silica gel G, Analtech Inc.) with chloroform:methanol:acetic acid:water (170:30:20:7 by vol.). Each lipid fraction was weighed, and the amounts of glycolipids were determined by the phenol-H_2SO_4 method[14] and those of phospholipids were determined according to Bartlett[15].

Analysis of fatty acids

Transesterification of fatty acids was carried out by incubating the lipids in anhydrous methanol containing 5% HCl for 3h at 100°C. Fatty acid methyl esters thus obtained were analyzed with a Shimadzu GC-7A gas chromatograph (Shimadzu Seisakusho Ltd.) equipped with a flame ionizing detector and a 0.3 x 200cm glass column filled with 15% DEGS on 80-100 mesh Chromosorb WAW (GL Science Co.). The chromatograph was run isothermally at 190°C and N_2 at a flow rate of 50ml/min was used as carrier gas.

Results

All the data in this report are the mean values and standard deviations of 3 measurements with 3 potato tubers (one measurement per potato). The statistically significant difference of the data was evaluated against unirradiated potatoes at 0 week (Control ; C) based on t-values. The lipid contents and fatty acid compositions of potato tubers one day after irradiation were almost the same as those immediately after irradiation, and the lipids of tubers one day after irradiation were analyzed as those of 0 week.

Lipid composition of potato tubers

The amounts of neutral lipids, phospholipids and glycolipids in irradiated potatoes were generally same as those of unirradiated ones 1 day after irradiation. The contents of neutral lipids and glycolipids of irradiated potatoes remained nearly constant at the level of unirradiated potatoes during storage at 5°C after irradiation (Fig.III–2).

However, the phospholipid content gradually increased for a few weeks after irradiation at 1kGy (Fig. III–2). The amounts of phospholipids in unirradiated potatoes were nearly constant during storage at 5°C ($p>0.1$) ; 44.3±2.8mg/100g for 0 week, 44.6±3.5mg / 100g for 1 week, 44.7±3.9mg / 100g for 2 weeks, 44.3±2.5mg / 100g for 3 weeks, 44.3±2.9mg / 100g for 4 weeks, 42.5±2.4mg / 100g for 6 weeks and 42.3± 2.1mg / 100g for 8 weeks. Phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) were identified as major lipids in the phospholipid fraction and the percentages of the three lipids in the phospholipid fraction were nearly constant irrespective of irradiation treatment and storage period (PE:28–31%, PC:40–43%, PI:13–15%, by mol.).

Esterified steryl glycosides (ESG), monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) were identified as major lipids in the glycolipid fraction and accounted for 22–25%, 26–27% and 43–47% (by mol.) of the fraction, respectively. The amounts of these three glycolipids were not influenced by irradiation or storage at 5°C.

Fatty acid composition of lipids

The fatty acid compositions of neutral lipids and phospholipids were not significantly influenced by irradiation ($p>0.1$). The amounts of linoleic and linolenic acids in glycolipid fraction did not change one day after irradiation, and stayed at the level of unirradiated potatoes. The amount, however, of linoleic acid decreased and that of linolenic acid increased in the glycolipid fraction when irradiated potato tubers were stored for a few weeks at 5°C (Fig.III–3), to which the changes in MGDG and DGDG greatly contributed (Fig.III–4). With the MGDG and DGDG linoleic acid decreased and linolenic acid increased depending upon radiation dose, while no dose dependent

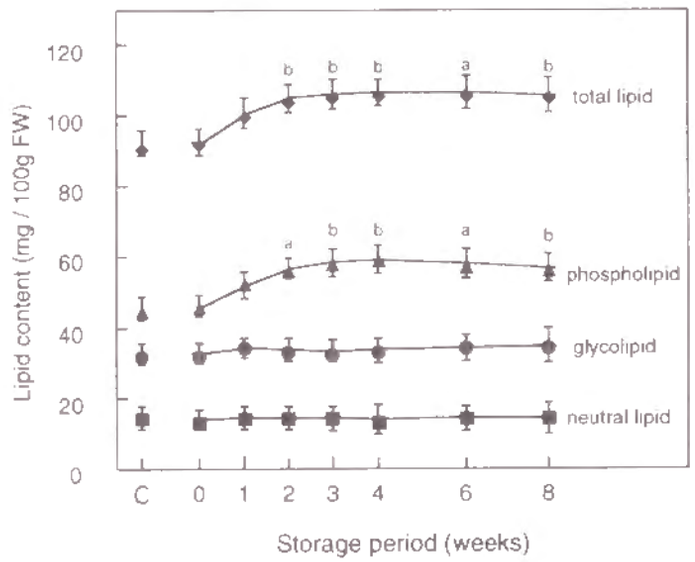

Fig. III - 2 Lipid Content of Irradiated Potato Tubers (1kGy) during Storage at 5°C.

■, Neutral lipid; ▲, phospholipid; ●, glycolipid; ◆, total lipid;
C, control (non-irradiated potato tubers at 0 week);
a, $P<0.01$; b, $P<0.05$; no symbol, $P>0.05$. The data of 1 day after the irradiation were defined as those of 0 week.

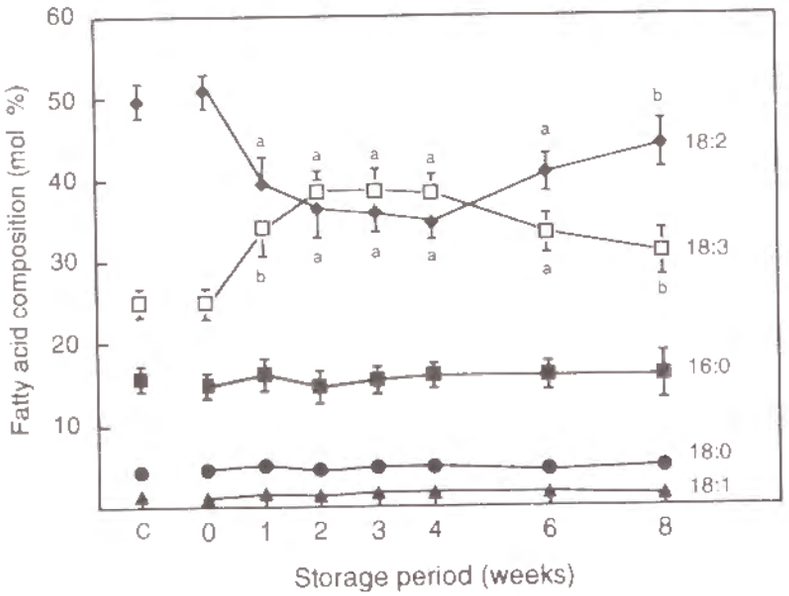

Fig. III - 3 Fatty Acid Composition of Glycolipid in Irradiated Potato Tubers (1 kGy) during Storage at 5°C.

□, palmitic acid; △, stearic acid; ▲, oleic acid; ■, linoleic acid; ●, linolenic acid; C, control (non-irradiated potato tubers at 0 week); a, $P < 0.01$; b, $P < 0.05$; no symbol, $P > 0.05$. The data 1 day after irradiation were defined as those of 0 week.

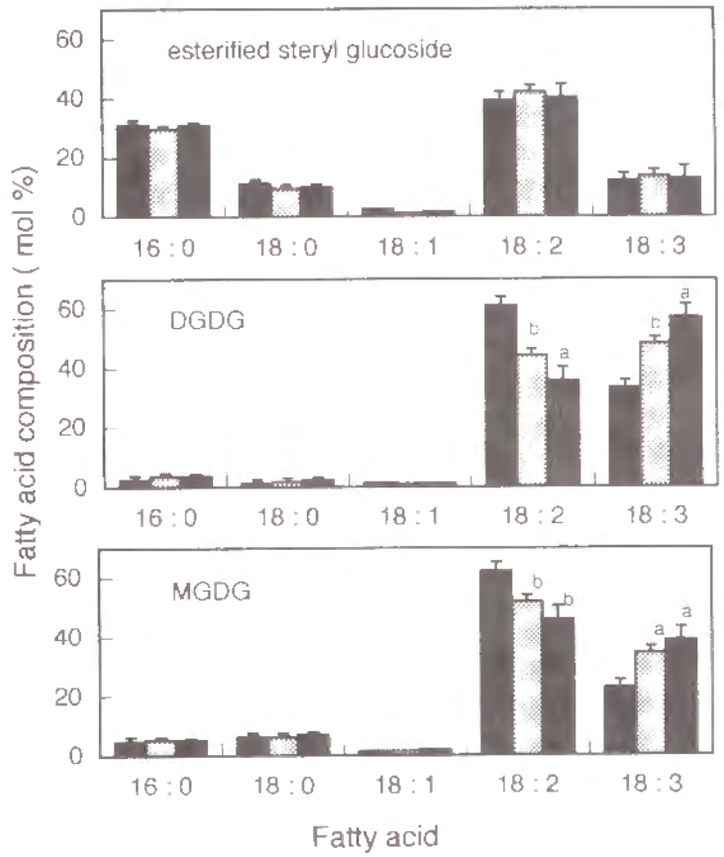

Fig. III - 4 Fatty Acid Composition of Major Glycolipids in Potato Tubers Stored for 3 Weeks at 5°C after Irradiation.

■, 0 kGy; ▨, 0.15 kGy; ■, 1 kGy; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid 18:3, linolenic acid; a, $P < 0.01$; b, $P < 0.05$; no symbol, $P > 0.05$.

change in the two fatty acids was observed in ESG (Fig. III-4). The amounts of these two fatty acids in the glycolipid fraction in unirradiated tubers did not significantly vary during storage at 5°C ($p>0.1$) ; the amounts of linoleic acid were 51.0±3.6% for 0 week, 49.8±3.2% for 1 week, 50.2±1.9% for 2 weeks, 49.7±4.0% for 3 weeks, 47.9±2.0% for 4 weeks, 48.1±3.5% for 6 weeks and 47.6%±2.6% for 8 weeks, and those of linolenic acid were 24.4+2.9% for 0 week, 24.8+1.8% for 1 week, 24.4+1.9% for 2 weeks, 25.5±3.2% for 3 weeks, 26.7±1.5% for 4 weeks, 26.8±1.9% for 6 weeks and 27.4±2.5% for 8 weeks.

Discussion

The composition of polar lipids of membranes in plants are influenced by various types of stress such as dehydration[16)-19)], osmotic stress[20)], low temperature [3),21-26)], fertilizer deficiency[27),28)], mechanical injury[4)] and senescence[29)]. The decrease in linoleic acid and the increase in linolenic acid in phospholipids or glycolipids have been observed in cold-stressed wheat[22),23)], rye[21),23)], apple[24)] and potato[3)25)]. The results on irradiated potato tubers in this study were similar to those on the cold-stressed plants in that the linoleic acid content decreased accompanied by an increase in linolenic acid content. Most of the MGDG and DGDG in potato tubers have been reported to be present in amyloplast membranes[30)]. The fatty acid compositions of MGDG and DGDG were significantly changed by gamma-irradiation in potato tubers in this study. These results suggest the possibility that the amyloplast membranes which play a role in starch-sugar interconversion are altered by irradiation.

III-3 Fatty acid composition of membrane fractions.

As mentioned above gamma-irradiation brought about the compositional changes of potato lipids during the storage depending on radiation dose; the linolenic acid (18:3) content of galactolipids increased, accompanied by the decrease in linoleic acid (18:2) content in galactolipids. These results indicated that membrane lipid was

changed in potato tubers, because lipids were mainly distributed in membrane fractions in potato tubers[31)]. However, no information about the lipid changes in individual organella membranes has been reported. In the present study membranes of irradiated tubers were fractionated into subcellular membranes and the compositional changes of fatty acid in each of the membrane fractions were investigated.

Materials and methods

Potato tubers

Potato tubers(Solanum tuberosom cv. Danshaku) were irradiated at 0.5 kGy in a Gamma-cell 220 (6.0kGy/h, AECL Canada) and stored at 5°C for 10 days after irradiation.

Preparation of membrane fraction

In order to investigate the effect of gamma-irradiation on each organelle membranes of potato tuber, cell membranes were fractionated by discontenious sucrose gradient. Two hundred g of potato tissues were homogenized in 400 ml of grinding medium with a vegetable juicer at 0 °C. The medium consisted of 0.25M sucrose, 75 mM 3-(N-Morpholino)propane sulfonic acid (MOPS), 5mM EDTA, 5mM Ethylene glycol-bisB-aminoethyl) ether (EGTA), 5mM Dithiothreitol (DTT), 2mM Phenyl methylsulfonylfluoride (PMSF), 1.5% Polyvinylpyrrolidone (PVP), 0.1% BSA and 1mg/ml 2,6-Di-tert.-buthyl-4-methylphenol (pH 7.8). The homogenate was squeezed through two layers of Miracloth and then subjected to differential centrifugations at 3000 x g for 10 min, 10,000 x g for 20 min and 100,000 x g for 60 min, consequently. The 10,000 x g pellet and the 100,000 x g pellet were designated as crude mitochondorial and microsomal fractions, respectively.

Two ml of the crude mitochondrial fractions (suspended in 0.25 M sucrose, 10 mM MOPS, 1 mM EGTA, 1mM DTT (pH 7.2)) were layered on to discontinues sucrose gradient consisting of 8.5ml each of 28, 36, 42 and 50% (w/w) sucrose solutions. Membrane loaded gradients were centrifuged for 2 h at 100,000 x g in an SW 28 rotor (Beckman). The protein band that occurred at the interface of 36 and 42 %

were collected and diluted with 0.25M sorbitol, 10mM MOPS, 1mM DTT and 1mM EGTA and then centrifuged for 40 min at 100,000 x g. The pellet thus obtained was designated as purified mitochondrial fraction (Mt).

The membrane fractions rich in plasmalemma (Pm), tonoplast (Tp) and endoplasmic reticulum (Er) were recovered from the crude microsomal fraction as follows. One point five ml of microsomal suspension are layered on to discontinuous sucrose gradient consisting of 8.5 ml each of 20 and 25 % (w/w) and 6ml each of 30, 34 and 38 % (w/w) sucrose solution and centrifuged in an SW28 rotor for 2h at 100,000 x g. The protein bands over the sucrose layers of 20, 30 and 38% were collected and designated as membrane fractions rich in tonoplast (Tp), endoplasmic reticulum(Er) and plasmalemma (Pm), respectively. The purity of each membrane fraction was determined by measuring the activities of the following enzymes as a specific membrane markers: antimycin insensitive NADPH–cytochrome c reductase, cytochrome c oxidase, vanadate sensitive ATPase (pH 6.5), nitrate sensitive ATPase (pH 8.0) and catalase[30].

The amyloplast membranes (Am) were isolated from potato tissue homogenate according to the method of Fishwick [30].

Lipids were extracted from each of the membrane fractions according to the method of Bligh and Dyer[8]. Methyl esters of membrane lipids were prepared and subjected to gas liquid chromatography according to the method previously described.

Results and discussion

Immediately after irradiation for membrane fractions of Pm, Tp, Er and Mt fraction the percentages of linolenic acid (18:3), were lower in irradiated tuber than those in unirradiated ones. However after storage for 10 days, the percentages of 18:3 were higher and those of 18:2 were lower in irradiated tubers as compared with unirradiated ones. As a result, in Pm, Tp, Er and Mt fractions, the ratios of 18:3 to 18:2 (18:3/18:2) were decreased immediately after irradiation and increased during storage for 10 days.

Table III–3.
Fatty acid compositions of plasmalemma(Pm), endoplasmic retucuram(Er), tonoplast(Tp), mitochandoria(Mt) and amyloplast(Am) in unirradiated (0kGy) and irradiated(0.5 kGy) potato tubers.

membrane fraction	0kGy	0.5kGy(0h)[a]	0.5kGy(10d)[b]
Pm			
16:0(mol%)	24.1	26.4	26.2
18:0	4.2	3.9	3.8
18:1	0.3	0.2	0.2
18:2	54.7	53.9	51.6
18:3	12.5	11.5	14.3
18:3/18:2	0.23	0.21	0.28
Tp			
16:0(mol%)	26.0	28.7	25.7
18:0	5.1	5.0	5.1
18:1	0.3	0.3	0.4
18:2	54.4	53.2	53.0
18:3	11.4	9.7	12.5
18:3/18:2	0.22	0.19	0.24
Er			
16:0(mol%)	19.2	19.6	20.4
18:0	3.8	3.5	3.9
18:1	0.2	0.3	0.2
18:2	59.5	59.7	54.4
18:3	15.0	13.4	18.0
18:3/18:2	0.25	0.23	0.33
Mt			
16:0(mol%)	17.1	17.9	17.5
18:0	2.9	2.5	2.3
18:1	0.3	0.3	0.4
18:2	63.7	63.9	62.5
18:3	14.1	13.0	15.3
18:3/18:2	0.23	0.20	0.25
Am			
16:0(mol%)	20.5	21.13	23.7
18:0	4.3	4.71	4.5
18:1	1.5	1.9	1.4
18:2	55.3	53.94	50.9
18:3	13.3	14.17	16.4
18:3/18:2	0.24	0.27	0.32

mean values of two measurements from two samples.

a Membrane fraction were prepared from potato tubers immediately after irradiation at 0.5 kGy.

b Membrane fraction were prepared from potato tubers 10 days after irradiation at 0.5 kGy.

In Am fractions of irradiated tuber, the percentage of 18:3 increased while 18:2 decreased immediately after irradiation. The 18:3/18:2 values elevated during storage for 10 days (table III–3). In our previous study, the increase of 18:3 content were mainly observed in the galactolipid moieties; monogalactosyl diacylglycerol(MGDG) and digalactosyl diacyl– glycerol(DGDG). In the case of potato tuber, plastid membrane is generally believed to be the site for the desaturation of 18:2 to 18:3[33], and amyloplast envelope of potato tuber was reported to be the site for the galactolipid synthesis[30]. The extent of increase of 18:3/18:2 by irradiation was larger in Am fraction than in other membrane fractions. The changes of FA composition in Am fraction support the postulation that the 18:3 increase in irradiated tuber is brought about by the desaturation of 18:2 to 18:3 in amyloplast membrane. On the contrary, in Mt fraction, galactolipids are the minor constituent of membrane lipids[34] and 18:3/18:2 increased to a smaller extent than in Am by irradiation.

The increase in 18:3/18:2 were observed in all the membrane fraction, which suggests that the exchange of membrane lipid among the organelles takes place actively in irradiated potato tuber as well as unirradiated ones[35].

III–4 Lipid content and fatty acid composition of irradiated potato tubers at different regions.

Generally chemical constituents such as sugars and enzyme proteins are not uniformly distributed in potato tubers[36], and the responses of potato tuber to some stresses have been observed to be varied with different parts of tubers[37),38]. It has been reported that the degree of sugar accumulation in potato tuber by cold storage gradually increased from stem end to bud end[37]. The enzymatic discoloration in potato tuber following cutting was higher in the stem end than the bud end[38].

It was expected that the susceptibility to radiation was different within a tuber. This study was undertaken to clarify the susceptibility of different part of potato tuber to radiation in terms of lipid contents: the contents and compositions of fatty acid in different regions of irradiated tuber were investigated.

Materials and Methods

Potato tuber

Potato tubers (cultivar Dejima) were irradiated at 0.5 kGy in a Gamma-cell 220 (6.2 kGy/h, AECL Canada) and stored for 3 and 28 days after irradiation at 5 °C in a dark room.

Tissues were taken from 6 different regions of one tuber according to the sampling pattern shown in Fig. III–5. The skin of tuber was removed to an approximate depth of 1mm. Tuber was divided into 3 sections; the apical (bud) region, basal(stem) region and central region, and each section was alphabetically labeled in that order. And each section was separated into outer (cortex) and inner (pith) layers.

Extraction and fractionation of lipids

Lipid extraction and fractionation were done by the methods previously described. Ten g of tissue were taken from each region of one tuber. Fifty g of the tissue from five tubers were boiled in isopropyl alcohol for five minutes, and lipids were extracted with chloroform–methanol. The lipids thus extracted were fractionated into neutral lipids, glycolipids and phospholipids with chloroform, acetone and methanol, respectively on a silicic acid column. After the solvents were evaporated into dryness, each lipid fraction was dissolved in a small portion of chloroform and stored under N_2 at –80°C.

Fatty acid analysis

Methyl esters of fatty acid were prepared from the neutral–, glyco–, and phospholipid fractions and fatty acid compositions of each fraction was determined by the method previously described.

Results and Discussion

Changes in fatty acid content of irradiated tuber

Fatty acids of total and phospholipid fractions were not uniformly distributed within tubers for both the control and the irradiated potatoes; outer layer (cortex tissue) contained higher amount of total and phospholipid fatty acid than inner layer (pith tissue). Lengthwise, the content of total and phospholipid fatty acid became higher in the order of central (C), basal (B) and apical (A) region (Fig. III–6). The large difference of fatty acid content between cortex and pith tissues is mainly attributed to the higher dry matter content of cortex tissue than pith tissue[39]. The greater

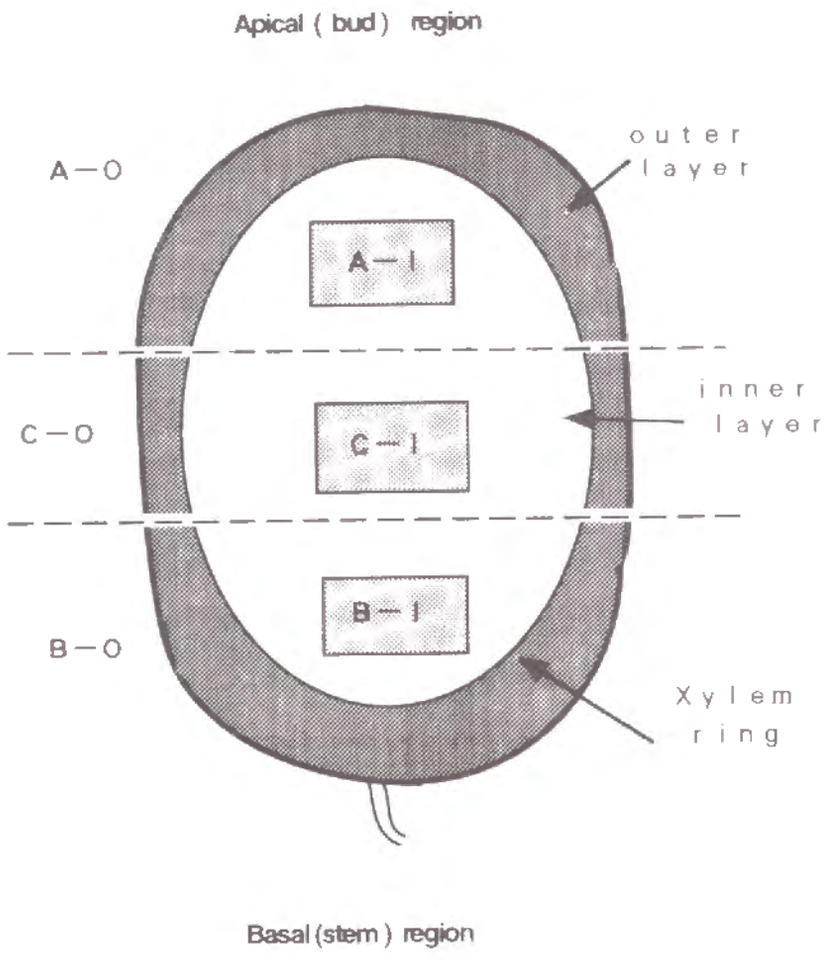

Fig. III - 5 Sampling Pattern of Tissues from Potato Tubers.

A - O : Apical (bud end) tissue of outer layer
B - O : Basal (stem end) tissue of outer layer
C - O : Central tissue of outer layer
A - I : Apical (bud end) tissue of inner layer
B - I : Basal (stem end) tissue of inner layer
C - I : Central tissue of inner layer

concentration of lipid in cortex tissue were also reported in previous investigations[38),40)]. Mondy and Muller [38)] reported that the amount of lipid in apical region was higher than that in basal region.

In the tissues of central region, both in outer (C-O) and inner layer(C-I) fatty acids in total and phospholipid fractions 3 days after irradiation were at lower levels than unirradiated tuber. However these values increased during 28 day storage. In apical (A) and basal (B) region, both in outer and inner layer, fatty acid amounts of total- and phospholipid fractions in the tubers 3 days after irradiation were slightly higher as compared with those in unirradiated ones. and further storage did not influence fatty acid levels (Fig III-6).

In the chapter III-1 the tendency of temporary drop of lipid content at 12 hours after irradiation, although it was not statistically significant. The temporary reduction of lipid content in central region after 3 days in the present study is consistent with our previous study, although the decline of fatty acid content in the other region was not observed in this experiment. Membrane lipid is continuously decomposed and regenerated in the cell[41)] and lipid content is determined by the final balance of lipolytic reactions and lipid synthetic reactions. Young immature tubers, which retain a higher capacity of cell division and membrane lipid synthesis, has higher lipid content than matured tubers [42)]. Even in matured tuber, a capacity of cell division is potentially retained and it appears in response to wounding, which is followed by the activation of fatty acid synthesis [43)]. It has been reported the capacity of wound healing is varied within tuber; cortex tissue shows higher rate of suberin formation than parenchyma tissue [44)]. The inconsistence of lipid change among different tissue regions 3 days after irradiation may be based on the different rate of metabolic reactions among the tuber regions. The induction of lipid synthesis may proceed more slowly in central region than the other region. As a result, decrease of fatty acid would be observed in central region at 3 days after irradiation. During a long storage period the fatty acid contents once decreased in central region elevated and the increased fatty acid contents were observed in any sampling region of irradiated tuber.

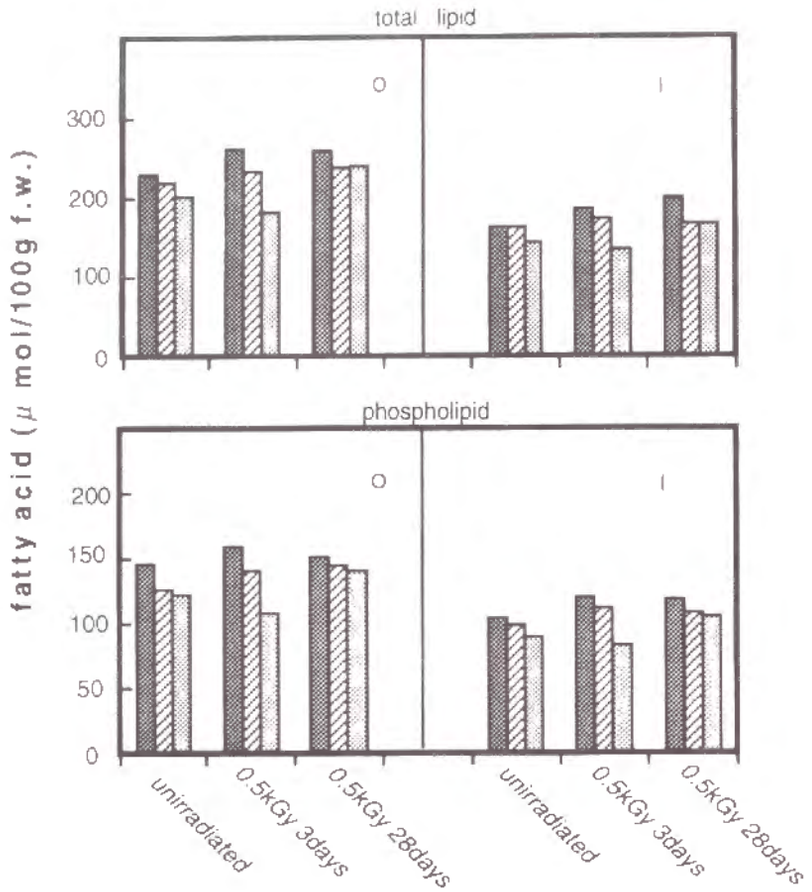

Fig. III - 6 Changes in Fatty Acid Contents of Total- and Phospholipid Fraction in Different Region of Irradiated Potato.

Fatty acid (μmole / 100g fresh weight) in outer(O) and inner(I) layer of region A, B and C from the tuber:

- : Apical region tissue (A)
- : Basal region tissue (B)
- : Central region tissue (C)

0.5 kGy 3days; tubers stored at 5 °C for 3 days after irradiation at 0.5 kGy
0.5 kGy 28days; tubers at 5 °C stored for 28 days after irradiation at 0.5 kGy

Changes in fatty acid composition

The percentages of saturated acids(palmitate and stearate) were not changed by irradiation. The sum of polyunsaturated fatty acid (18:2+18:3) for each regions was almost 70% of total fatty acids regardless of irradiation treatment and storage.

table III-4
Changes in linoleic acid(18:2) and linolenic acid(18:3) contents and the ratios of linoleic acid to linolenic acid(18:3/18:2).

tissue regions[a]	control	0.5kGy(3day)	0.5kGy(28day)
18:2 (umole/100gFW)			
A-I	83.0	96.2	93.6
A-O	102.3	114.3	105.1
B-I	84.7	87.7	78.6
B-O	100.1	99.9	100.1
C-I	75.8	68.1	80.0
C-O	94.7	78.1	101.4
18:3 (umole/100gFW)			
A-I	28.7	37.3	43.3
A-O	58.2	73.6	76.1
B-I	27.4	32.3	37.5
B-O	51.1	63.6	67.6
C-I	25.0	25.0	34.1
C-O	46.5	49.3	65.4
18:2+18:3 (% of total acid)			
A-I	70.4	72.8	69.5
A-O	70.7	72.6	70.9
B-I	69.6	70.3	69.3
B-O	69.9	71.1	71.2
C-I	71.0	70.3	69.7
C-O	71.8	71.2	70.6
18:3/18:2			
A-I	0.34	0.39	0.46
A-O	0.57	0.64	0.72
B-I	0.32	0.37	0.46
B-O	0.51	0.64	0.68
C-I	0.33	0.37	0.43
C-O	0.49	0.63	0.65

a: tissues were sampled as shown in Fig.III-5

The contents of linoleic acid (18:2) (μ moles / 100g F.W.) were lower in central region (C-O, C-I) 3 days after irradiation, while in other region of irradiated tubers even at 3 days and 28days after irradiation the contents (μ moles /100g F.W.) of linoleic acid (18:2) were almost same or higher as compared with unirradiated tubers. In all the

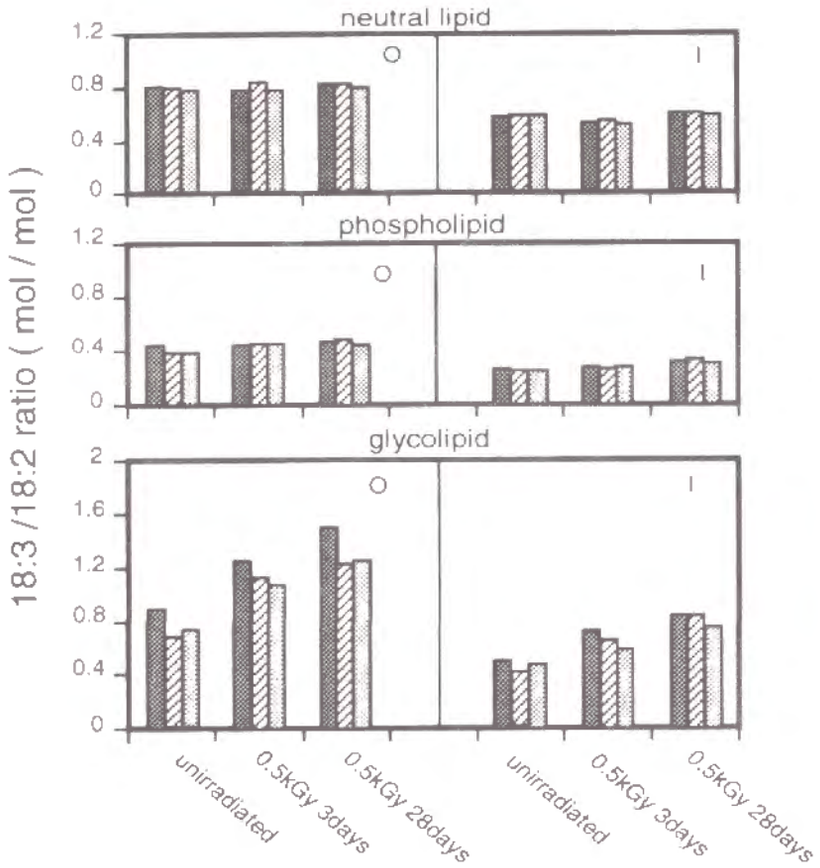

Fig. III - 7 Linolenic Acid to Linoleic Acid Ratio (18:3/18:2) of Each Lipid Fractions.

Mole ratio of linolenate to linolate in neutral-, phnspho- and glyco-lipid of each region of the tuber.

0.5 kGy 3 days; tubers stored at 5°C for 3 days after irradiation at 0.5 kGy.
0.5 kGy 28 days; tubers stored at 5 °C for 28 days after irradiation at 0.5 kGy.

■ : Apical region tissue (A)
▨ : Basal region tissue (B)
▩ : Central region tissue (C)

O : outer layer
I : inner layer

tissues of irradiated tubers the contents of linolenic acid(18:3) increased, and the mole ratio of 18:3 to 18:2 (18:3/18:2) were significantly elevated during the storage (Table III-4).

This increase in linolenic acid is mainly attributed to the changes of fatty acid composition in the glycolipid fraction (Fig.III-7) and these results were consistent with our previous ones on whole irradiated tubers (Chapter III-2).

In the present study, different responses of lipid content, both increase and decrease of lipid, were observed in irradiated tuber depending on the region of tuber and storage period after irradiation. After a long period of storage, lipid contents were at higher levels in any region of irradiated tuber as compared with unirradiated one, and the increased ratio of linolenic acid to linoleic acid was observed regardless of tuber region.

III-5 Effects of gamma-irradiation on lipid metabolic changes of potato tubers in response to mechanical injury

It has been reported that mechanical injury causes membrane lipid breakdown in potato tubers, followed by membrane lipid synthesis.[4] This reconstitution of biological membranes has been considered to be essential for the development of wound respiration.[5]

Gamma-irradiated potatoes have been reported to become susceptible to microbial infection.[45,46] It has been reported that the degree of wound-induced changes, such aspolyphenol synthesis,[47,48] induction rate of the isozymes of peroxidase,[49] development of wound healing,[50] and formation of phytoalexins[51] are lowered by irradiation.

Since polymeric materials of wound periderm (suberin) of potato tuber slices consist of lipids[52] and phenolic compounds,[53] lipids are expected to be involved in development of wound healing. The metabolism of lipids of potato tubers in response to injury would be influenced by irradiation.

This study was undertaken to investigate the effects of gamma-irradiation on the lipid changes in response to mechanical injury, especially on fatty acid synthesis and desaturation.

Materials and methods

Potato tuber

Two cultivars of potato (Solanum tuberosum), Dejima grown in Nagasaki and Danshaku in Hokkaido, were used in this study.

Irradiation of potatoes

Potato tubers were irradiated at 0.5 kGy in a Gamma-cell 220 (AECL Canada, 6.2 kGy/h). After irradiation potatoes were stored at 5°C in a dark room.

Wounding and [^{13}C]acetate administration to potato tubers

Nine punctures(4 cm in depth, 0.5 mm in diameter) were made with a needle of a microsyringe in the central region (6 mm x 6 mm) of potato tubers, and 4 μl of 1.5 M of sodium [^{13}C] acetate or 20 mM phosphate buffer (pH=6.5) was applied to each puncture. Tubers were incubated at 25°C for 24 h, followed by the extraction of lipids, unless otherwise stated.

Extraction and fractionation of lipids

Lipids extraction and fractionation were done by the methods previously described Ten g of pulp tissue were taken from the wounded area of one tuber. Fifty g of the tissue from five tubers were boiled in isopropyl alcohol for five minutes, and lipids were extracted with chloroform-methanol. The lipids thus extracted were fractionated into neutral lipids (NL), glycolipids (GL) and phospholipids (PL) on a silicic acid column. Each lipid fraction was dissolved in a small portion of chloroform and stored under N_2 at -80°C.

Preparation and fractionation of fatty acid methyl esters

Methyl esters of fatty acid were prepared from the NL, GL, and PL fractions by the method previously described. The methyl esters prepared were fractionationed depending upon the degree of unsaturation by silver-ion column chromatography by the method of Criste[54], as follows. Methyl esters were put on a solid-phase extraction column (Bond elut SCX, Analyticalchem Inc.) which had been first converted to the silver ion form. The fractions containing saturated, monoenoic, dienoic, and trienoic fatty acid methyl esters were eluted with hexane:ether (95:5 v/v), hexane:either (72:28), ether:acetone (90:10), and acetone:acetonitrile (80:20), respectively.

^{13}C analysis

The ^{13}C in the lipid fractions and fatty acid (FA) fractions was measured with an infrared $^{13}CO_2$ analyzer with a combustion system (Model EX-130S, Japan Spectrometric Co.Ltd.). Thirty to fifty microliters of lipid in chloroform solution or FA in hexane solution,which contained 100-300 μg total carbon, was put into a quartz glass cup and gently warmed to remove the solvent completely. The sample cup fell into a combustion furnace where the sample was immediately oxidized at 900°C under a continuous O_2 gas flow. Carbon dioxide evolved was introduced with carrier gas O_2 to an infrared analytical cell. The absorption intensity of infrared radiation was measured at two different infrared regions, 2390 to 2370 cm^{-1} and 2280 to 2260 cm^{-1}. The ^{13}C and ^{12}C in the sample were measured from the absorbance at 2390-2370 cm^{-1} and 2280-2260 cm^{-1} with calibration curves obtained with ^{13}C labeled glycine (99 atom% ^{13}C) and unenriched glycine (1.11 atom% ^{13}C) by the method of Okano et al.[55] The ^{13}C incorporated into lipid fractions from [^{13}C] acetate was calculated as follows:

$$^{13}C \text{ incorporated} = (^{12}C + ^{13}C) \times (^{13}C_{enrich} - ^{13}C_{natural}) \times (1/100)$$

where:

$(^{12}C + ^{13}C)$: total carbon (μmole) in sample

$^{13}C_{enrich}$: abundance of ^{13}C (atom%) in enriched lipid sample

$^{13}C_{natural}$: abundance of ^{13}C (atom%) in unenriched lipid sample

abundance of ^{13}C : $(^{13}C / (^{12}C + ^{13}C)) \times 100$ (atom%)

Chemicals

Sodium [U-^{13}C] acetate(99 atom% ^{13}C) was obtained from Cambridge Isotope Laboratories. All the other chemicals used were analytical reagent grades.

Results and discussions

Fatty acid changes in irradiated and punctured tubers

For both cultivars of potatoes, either irradiation or puncturing did not cause significant changes in the fatty acid composition of NL and PL (data are not shown). In GL fractions the percentages of both saturated fatty acids, palmitic acid (16:0) and

stearic acid (18:0), were not altered by irradiation or puncturing treatment.

Figure III-8 shows the amounts of linoleic (18:2) and linolenic (18:3) acids in GL fractions of gamma-irradiated and punctured tubers. For both Dejima and Danshaku potatoes, these polyunsaturated fatty acids amounted to about 70% of total fatty acids, and the percentage of (18:2 + 18:3) was almost the same in irradiated and punctured tubers (Fig.III-8a). Irradiation caused an the increase of the 18:3 content and a decrease in the 18:2 content for non-punctured tubers increased to a higher level than that in unirradiated ones depending on the storage period after irradiation (Fig.III-8b). This unsaturation was observed in both cultivars, Danshaku and Dejima, and these results agree with the results previously reported.

It has been reported that slicing a potato tuber, another type of mechanical injury, causes metabolic changes related to fatty acid unsaturation; synthesis of polyunsaturated fatty acid[56] and induction of fatty acid desaturase[57]. It is obvious that the increase in 18:3/18:2 of GL fraction in potato tubers administered with buffer or acetate solution is attributable to the mechanical stress by puncturing.

The values of 18:3/18:2 of unirradiated tubers were significantly enhanced by the administration of buffer or acetate solution for both cultivars. The increase in 18:3/18:2 value caused by the administration of buffer or acetate solution was smaller in the tubers stored for a short period after irradiation (2.5 days). When irradiated tubers were stored for a longer period (28 days), the response of fatty acid to the puncturing became larger; which suggests that the response to injury which was damped by irradiation and was restored during storage for longer periods.

Incorporation of ^{13}C into lipid fractions

Figure III-9 shows the course of ^{13}C(μmole) incorporation into NL,GL,and PL fraction from [^{13}C] acetate in tubers of var. Dejima. The ^{13}C was incorporated into PL in unirradiated tubers faster than the irradiated sample and reached the maximum value after 24 h, then gradually decreased. The ^{13}C content of PL in the irradiated tubers that were stored for 2.5 days after irradiation increased for 72 h. The ^{13}C level of NL in unirradiated tubers significantly increased within 24 h, and then decreased. The level of ^{13}C of NL in irradiated tubers gradually increased for 48 h, and then slightly declined. The rate of ^{13}C incorporation into GL fraction was rather small as

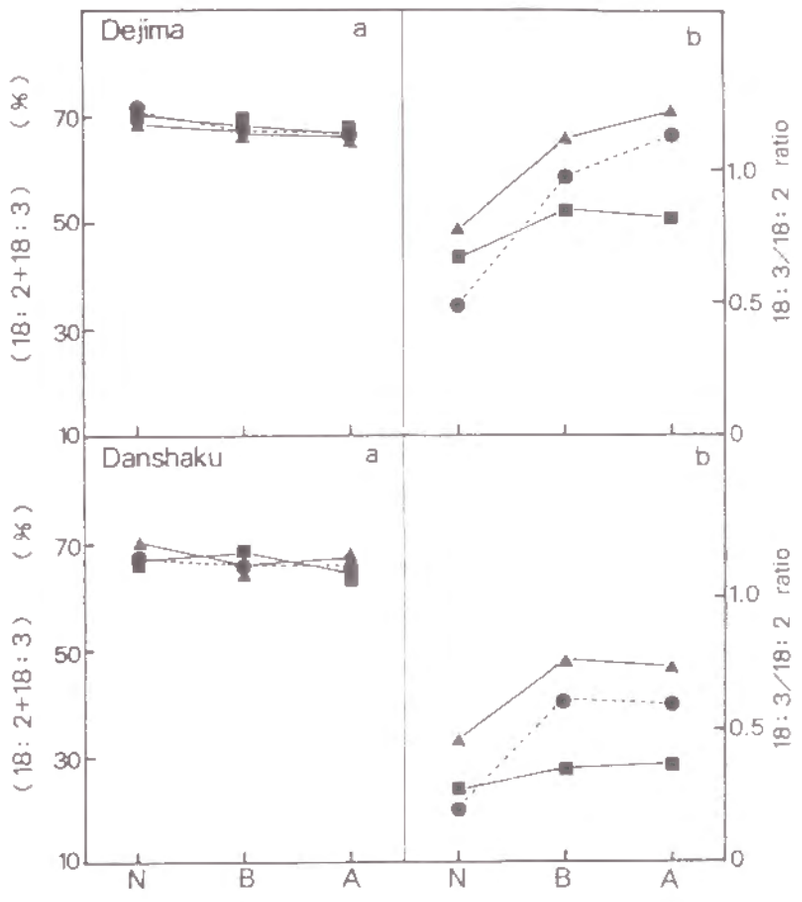

Fig. III - 8 Amounts of Linoleic Acid (18:2) and Linolenic Acid (18:3) in Glycolipids Fractions in Gamma-irradiated and Punctured Tubers.

a) percentage of linoleic acid and linolenic acid (18:2+18:3)(mol%); b) the ratio of linolenic acid to linoleic acid (18:3/18:2). N, without puncturing treatment; B, incubated at 25°C for 24h after puncturing and administration of phosphate buffer; A, incubated at 25°C for 24h after puncturing and administration of sodium acetate solution. ●, unirradiated tuber; ■, irradiated at 0.5 kGy and stored 2.5 days at 5°C before puncturing; ▲, irradiated at 0.5 kGy and stored 28 days at 5°C before puncturing.

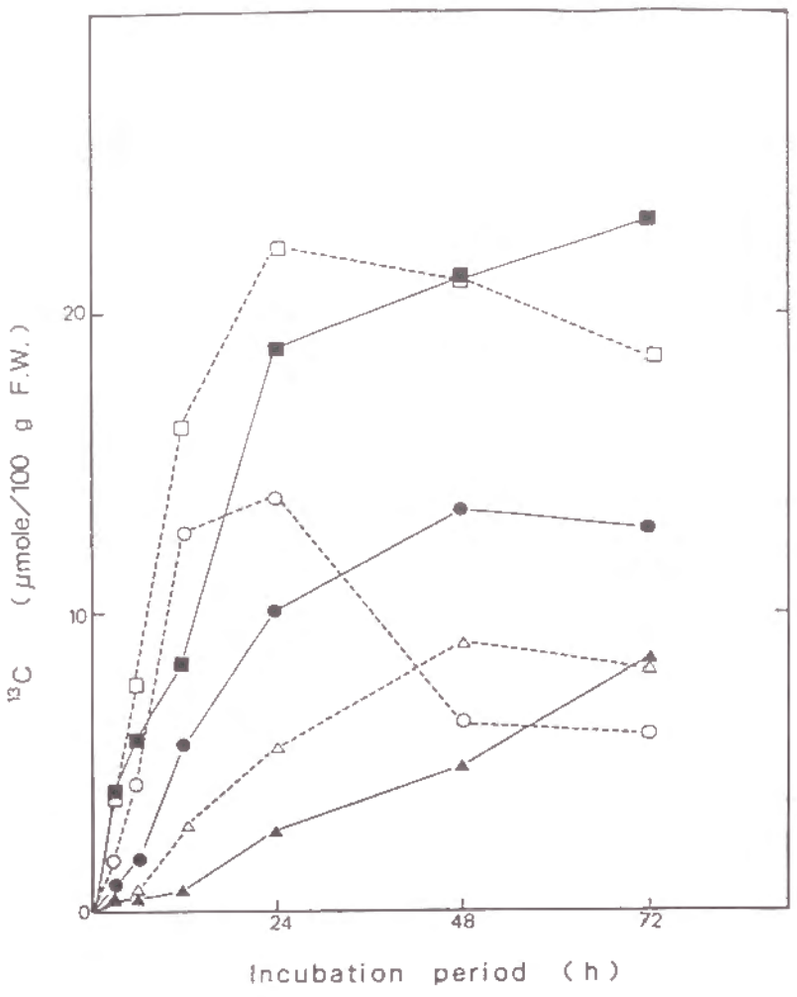

Fig. III - 9 Incorporation of ^{13}C-acetate into Lipid Fractions of Potato Tubers after Gammma-irradiation.

Potatoes irradiated at 0.5 kGy were stored for 2.5 days at 5°C and then treated with [^{13}C] acetate. ○, 0 kGy Neutral lipids; ●, 0.5 kGy Neutral lipids; □, 0 kGy Phospholipids; ■, 0.5 kGy Phospholipids; △, 0 kGy Glycolipids; ▲, 0.5 kGy Glycolipids.

compared with the other two lipid fractions. The elevation of ^{13}C level of GL in irradiated tubers was slower than that in unirradiated tubers. However, the level of ^{13}C in the irradiated sample reached the same level as unirradiated tubers after incubation for 72 h. The incorporation of ^{13}C from ^{13}C labeled acetate into lipid fractions of unirradiated tubers after puncturing may reflect the induction of fatty acid synthetic activity by mechanical injury, which has been reported for sliced potatoes based on the experiment on incorporation of ^{14}C acetate.[58] The results shown in Fig. III-9 suggest that the [^{13}C] acetate was more slowly metabolized in irradiated tubers as compared with unirradiated tubers.

Incorporation of ^{13}C into each fatty acid of lipid fractions.

Figure III-10 shows the distribution of ^{13}C label in the fatty acid of lipid fractions. In the NL and PL fractions of irradiated tubers, saturated fatty acids were highly labeled during the incubation at 25°C, and ^{13}C incorporation into the dienoic FA fraction proceeded slowly as compared with unirradiated tubers. In unirradiated tubers ^{13}C were incorporated into the dienoic acid fraction of NL to a greater extent than the other fatty acids and both saturated and dienoic acids of PL were labeled at the same rate. In both irradiated and unirradiated tubers trienoic fatty acid fraction was weakly labeled in the PL fraction. These results indicated that irradiation retarded the desaturation to dienoic acids from saturated ones in some processes, although the retardation was not clearly observed in the compositional changes of unlabeled fatty acids of PL and NL fractions. In potato tubers it was reported that the desaturation of oleic acid to linoleic acid took place in the molecule of phosphatidylcholine (PC), and oleoyl-PC desaturase was induced on microsomes of potato slices during aging[59]. Therefore, the low extent of ^{13}C incorporation into the dienoic acid fraction in PL of irradiated tubers suggests some effects of the irradiation on the development of this desaturase. For the GL fraction the ^{13}C incorporation into di and tri-enoic acids proceeded more rapidly in unirradiated tubers than in irradiated ones. The results on the incorporation of ^{13}C label into fatty acids of GL are consistent with those shown in Fig.III-8; the increase of 18:3/18:2 value by puncturing was smaller in irradiated tubers than that in unirradiated ones. However further study will be necessary to clarify the direct effects of irradiation on the desaturation of linoleic acids to linolcic acid in punctured tuber.

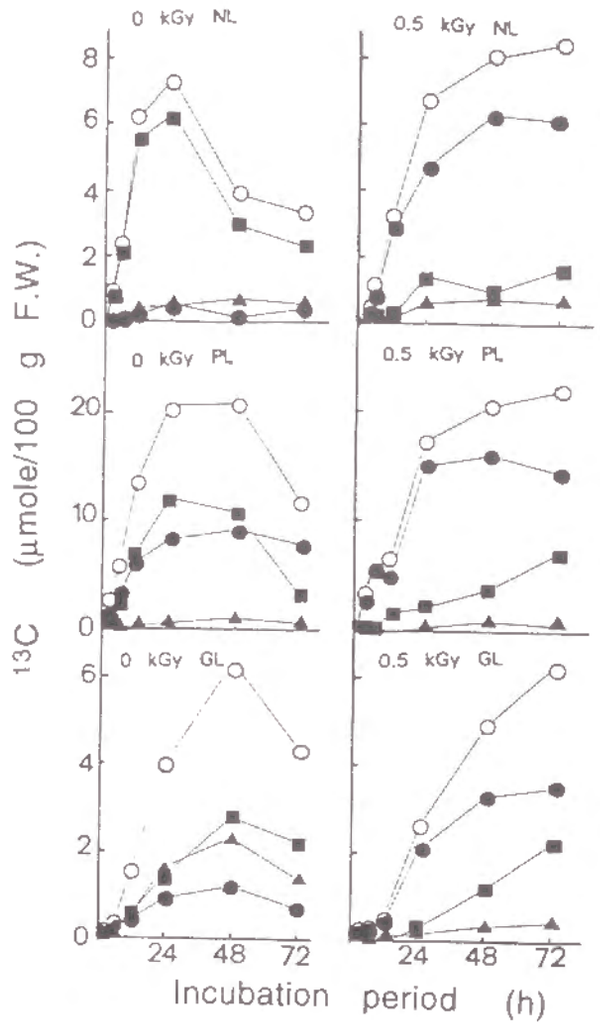

Fig. III - 10 ^{13}C Incorporation into Fatty Acids of Neutral Lipids (NL), Phospholipid (PL), and Glycolipid (GL) in Potato Tubers.

Potatoes irradiated at 0.5 kGy were stored for 2.5 days 5°C and then used in the experiment. ●, saturated fatty acid fractions; ■, dienoic fatty acid; ▲, trienoic fatty acids; ○, sum of saturatied, dienoic, and fatty acid.

The results in this study indicate that irradiation reduces the extent of the response of potatoes to mechanical injury in terms of not only polyphenol metabolism[47-50] and phytoalexin formation[51] but also lipid metabolims. Irradiation retarded fatty acid desaturation as well as fatty acid synthesis induced by mechanical injury.

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Chapter IV

Physicochemical properties of membranes of gamma-irradiated potato tubers

Lipid bilayer of biological membrane provides the basic structure of membrane and serves as a relatively impermeable barrier to the flow of most water soluble molecules. The protein molecules are dissolved in lipid bilayers and mediated the various function of the membrane. The activity of certain membrane bound enzyme is dependent to an great extent on the lipid constituents of biological membrane [1]. Ionizing radiation has been reported to deteriorate membrane integrity, which results in the damage of permeability and inactivated membrane bound enzymes as reviewed by von Sonntag[2]. This chapter dealt with the physicochemical characterization of membranes and inactivation of membrane bound enzyme in gamma-irradiated potato tubers.

IV- 1 Electrical impedance of gamma-irradiated potato tubers

The electrical impedance or conductivity of potatoes determined by puncturing tuber with electrode was altered by irradiation in dose dependent relation [3]. An alteration in the impedance of plants has been ascribed to changes in membrane. Fujii [4] et al. reported to changes in impedance of tomato seedling varied in accordance with changes in their polar lipid composition. As described in chapter III, lipid distribution and its compositional changes by gamma-irradiation varied with in the tuber. It was postulated that the alteration of electrical impedance varied within tubers in accordance with lipid changes by gamma-irradiation. In this study electrical impedance was measured in different regions of potatoes.

Materials and methods

Irradiation and storage of potatoes

Potatoes of cultivars of Danshaku (harvested in Shihoro), Dejima(Nagasaki), and May-Queen(Aikoku) were irradiated at 100 ±25Gy in a Gamma-cell 220 (6.0kGy/h, AECL). Both irradiated and unirradiated potatoes were stored at 5 °C in a dark room.

impedance measurement of potatoes

Impedance of potatoes was measured under the following condition. Before the experiments potatoes were adjusted to 22°C and electrical measurement were done at that temperature with stainless-steal two electrode system: 1 mm diameter, 10 mm long, and 10 mm apart. A potato tuber were punctured with the steel electrode connected to a Digital Spectral Analyzer TR9403 (Advantest Ltd.). Alternating current was measured. Impedance parameter were calculated from the current and voltage and expressed as follows. All the mean values and standard deviations were from 5 measurements in 5 potato tubers.

Z_{5k} : magnitude of impedance at 5 kHz

Z_{50k}: magnitude of impedance at 50 kHz

Results and discussion

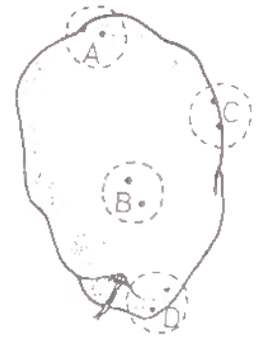
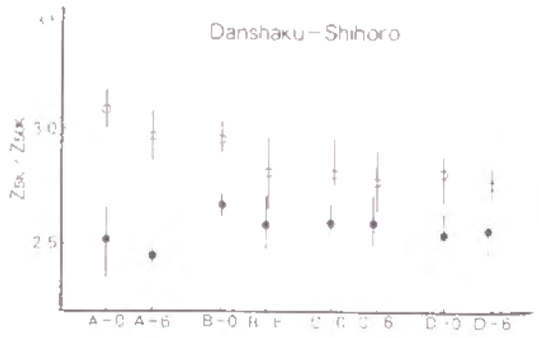
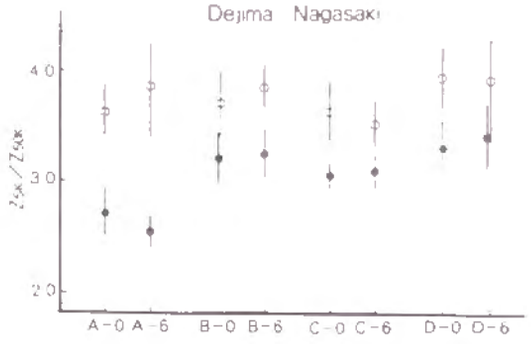


Fig.IV-1 Regions of Potato Tuber for Impedance Measurement

Impedance was measured at 4 different regions (A, Apical; B, Central; and C, Side and D,Basal) (Fig. IV-1) for three varieties of potatoes. The impedance ratio, Z_{5k}/Z_{50k}, measured at the apical region showed the largest difference between unirradiated and irradiated potatoes, irrespective of potato cultivars (Fig. IV-2). The impedance ratio measured at an apical region was almost constant during storage after irradiation and allowed differentiation between unirradiated and irradiated potatoes for up to 6 months. The impedance ratio for unirradiated potatoes of Danshaku cultivar was from 2.4 to 2.7 and that for irradiated potatoes was from 2.8 to 3.2 irrespective of harvesting locality. The parameters for unirradiated products were 2.5-2.9 and 2.7-3.0 and those for irradiated products were 3.5-4.1 and 4.5-5.5, for Dejima and May-Queen, respectively.





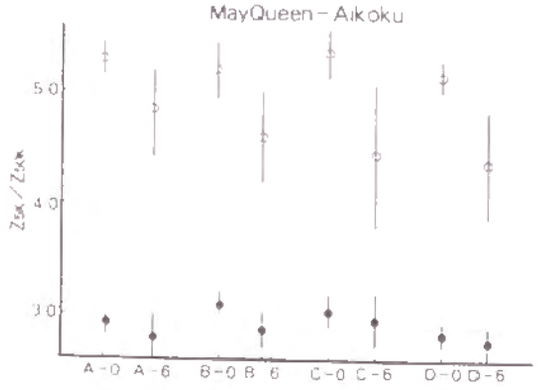


Fig. IV - 2 Z_{5K}/Z_{50K} at Different Regions of Potato Tubers.

●, 0 Gy; O, 100 Gy. A, Apical; B, Central; C, Side; D, Basal. A-0, impedance ratio at apical region 1 week after irradiation; A-6, impedance ratio at apical region 6 months after irradiation; B-0, impedance ratio at central region 1 week after irradiation; B-6, impedance ratio at central region 6 months after irradiation; C-0, impedance ratio at side region 1 week after irradiation; C-6, impedance ratio at side region 6 months after irradiation; D-0, impedance ratio at basal region 1 week after irradiation; D-6, impedance ratio at basal region 6 months after irradiation.

It has been reported that the cellular conditions, especially conditions of cellular membranes influenced electrical impedance of biological tissues [5),6)]. As shown in chapter III, the content of polar lipid and the extent of phospholipid increase by irradiation were higher at apical region than basal region. The fact that the apical region of a tuber showed the largest impedance difference between unirradiated and irradiated potatoes may suggest that the impedance measurement detected an alteration in cellular membranes brought about by irradiation.

IV-2 Membrane permeability of potato gamma-irradiated potato tubers

Irradiation has been reported to influence membrane permeability of erythrocytes [7),8)] and microorganisms [9)-11)]. An enhanced rate of leakage of ions was observed in irradiated carrots [12)].

It was postulated that the membrane permeability of potato tubers would be influenced by irradiation. In this study, the leakage of electrolytes from disks of irradiated potatoes was determined and results were discussed with respect to lipid changes in potato tubers caused by irradiation.

Materials and methods

Potatoes

Potatoes (Solanum tuberosum cv.Dejima) grown in Nagasaki were used in this study. Prior to irradiation, potato tubers were stored for one month at 5°C after harvest.

Irradiation of potatoes

Potatoes were irradiated at 1kGy in a Gamma-cell 220 (AECL, 6.2kGy/h) unless otherwise stated. After irradiation potato tubers were stored at 5°C in a dark room.

Determination of membrane permeability of potatoes

The membrane permeability was expressed as the rate of leakage of electrolytes from potato as follows; Ten disks (25mm diameter, 5mm thick) taken from midparenchyma of one tuber were rinsed with distilled water and wiped with filter paper. The 10 disks thus prepared were incubated at 27°C in 100ml of distilled water

under a gentle shake, and the electrical conductivity of the water was measured at 27°C with a Conductivity Meter ES-12 (Horiba Ltd.) at 15min (C_{15}) and 75min (C_{75}). The disks were then frozen (-80°C) and thawed, followed by incubation for 1h at 27°C in the water. The electrical conductivity of the water was measured to determine total electrolytes (C_t). The rate of electrolyte leakage (%/h) was expressed as $100 \times (C_{75}-C_{15})/C_t$.

Results and Discussion

All the data in this report are the mean values and standard deviations of 3 measurements with 3 potato tubers (one measurement per potato). The statistically significant difference of the data was evaluated against unirradiated potatoes at 0 week (Control ; C) based on t-values.

The rate of leakage of electrolytes from the disks of irradiated potatoes increased immediately after irradiation and then continued to increase for two days, followed by a gradual decrease (Fig. IV-3). The rate of leakage from the disks of unirradiated potatoes remained almost constant during storage at 5°C. Higher radiation dose (1kGy) resulted in a higher rate of leakage of electrolytes from the disks.

In this study the rate of leakage of electrolytes from potato disks increased within 1h after irradiation. Scherz[13] reported that the change in impedance of potato tubers was clearly observed within 6 h after irradiation. The increase in the rate of leakage of electrolytes as well as the changes in the impedance value determined by puncturing potatoes with electrodes[3] exhibited a dose dependent relation. Given the enhanced leakage of electrolytes and the change in impedance, it is obvious that some alteration of membranes in potato tubers occurs in a short period after irradiation. It has been reported that accumulation of lipid hydroperoxide [14] and FFA[15] stimulated the membrane permeability in model systems. From the results of previous chapter, the possibility of the accumulation of hydroperoxide by irradiation of potato tuber was negligible. Although the increase in FFA content was not significant, the period when the slight increase in FFA/TFA was observed after irradiation was almost the same as the period when electrolyte leakage significantly increased. The FFA level decreased to

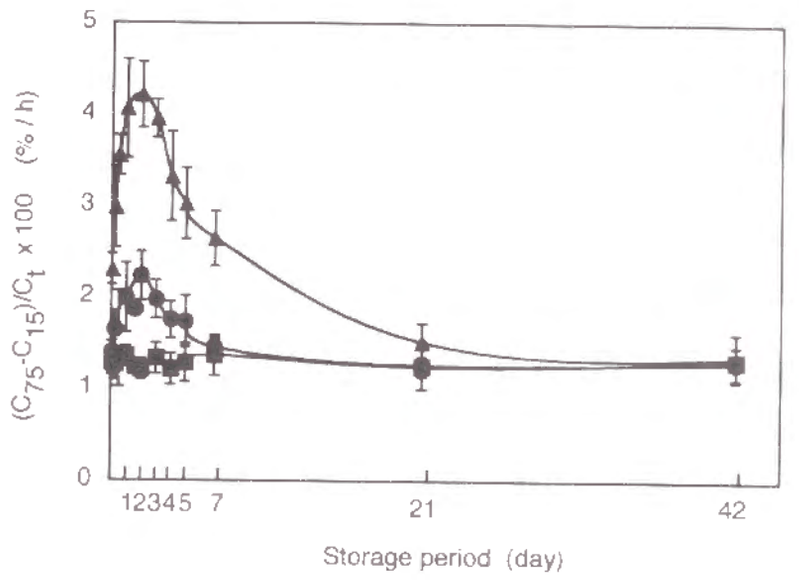


Fig. IV - 3 Electrolyte Leakage of Potato Disks. (Means ± S.D)

Electrolyte leakage was caluculated by;

$$\text{Electrolyte leakage (\% / h)} = 100 \times (C_{75} - C_{15}) \ / \ Ct$$

where Ct: Conductivity of water with freeze-thawed potato disk (total electrolyte leakage)
C_{15}: Conductivity of water incubated with potato disks for 15 min.
C_{75}: Conductivity of water incubated with potato disks for 75 min.
■, 0 kGy; ●, 0.15 kGy; ▲, 1.0 kGy

original level within 3 days followed by the increase in the phospholipid content and fatty acid unsaturation in galactolipids, which is concomitant with the recovery of the damage of membrane permeability. These lipid changes may contribute to the recovery of membrane integrity shown in electrolyte leakage.

IV-3 Effect of gamma-irradiation on the activity of tonoplast H^+-ATPase from potato tubers

The vacuoles in higher plant cells are essential for the maintenance and regulation of the homeostatic environment of cytoplasm. It participates in transport and storage of ions and sequesters metabolyte and lytic enzymes[16]. Clearly many of these activities depend on the properties of the vacuolar membrane, tonoplast. An electrogenetic H^+-ATPase present in tonoplast provides proton motive force for the active transport of solute across vacuolar membrane [17][18]. Therefore tonoplast H^+-ATPase has been attracted the attention in the study of environmental stresses in plants. DuPont and Mudd [19] found proton transport by tonoplast vesicles to be closely correlated to cold acclimation. The activity of tonoplast ATPase in cultured cell of chilling-sensitive rice [20], and mung bean hypocotyls [21][22] was decreased by the exposure to low temperature. In salt tolerant plant, Atriplex gmelini, the activities of tonoplast H^+-ATPase increased by the treatment with high concentration of Na, which provided the driving force to accumulate Na in vacuoles [23]

Radiation effects on biological membranes, in general, have been the subject of the investigation [24]. However at present, a little biological data are available on plant materials [12],[25]-[27]. Although Sato et al.[27] have reported the damage of latex vacuolar membranes by gamma-irradiation, the information about the radiation effects on vacuolar membrane, paticuraly present in plants, is lacking.

In Chapter VI-2, it was shown that electrolyte leakage from potato tuber tissue increased immediately after gamma-irradiation, which implies that the integrity of plasmalemma and tonoplast of potato tuber may be lost by gamma-irradiation.

This study was undertaken to clarify the effect of radiation on tonoplast H^+-ATPase activity in potato tuber. Inactivation of tonoplast H^+-ATPase was amplified

by degradation of the tonoplast lipid with gamma-irradiation, especially, the accumulation of FFA in tonoplast during postirradiation period.

Materials and methods

Potato tuber

Potato tuber(solanum tuberosum cultivar Waseshiro) were purchased from a local market in Tsukuba and stored at 5 °C in a dark room for about 1 month before starting of experiments.

Isolation of tonoplast-enriched membrane

Tonoplast vesicles were isolated as described by Kasamo[28] with a slight modification (Fig. IV-4). In brief, 25 g of potato tissue was taken from one tuber and 50 g of tissues from two different tubers were ground with a chilled kitchen grater in 100 ml of grinding medium that containing 0.25 M mannitol, 5 mM EGTA, 5 mM EDTA, 5 mM DTT, 1.5%(w/v) polyvinylpyrrolidone, 0.1% BSA(w/v), 0.001% BHT and 50 mM Mops/KOH (pH 7.8). After filtration through 2 layers of gauze, the homogenate was centrifuged at 3,000 x g for 10 min. The supernatant was centrifuged at 10,000 x g for 30 min. The precipitate was suspended in 0.6 ml of suspending buffer containing 0.25 M mannitol, 10 mM Mes-Tris (pH 7.3), 1 mM EGTA, and 1 mM DTT. 0.2 ml of suspension was layered over a dextran step gradient composed of 4.5ml of 6% dextran (dextran T-70 Pharmacia Fine Chemicals, Uppsala Sweden) (bottom) in suspending buffer and 4ml of 0% dextran (top) in the same suspending buffer. The step gradient was centrifuged for 1.5h at 120,000 x g in a Beckman SW 40 rotor. The interface between 0 and 6% dextran was collected with a Pasteur pipette and diluted five-fold with 0.25M mannitol containing 10 mM Mes-Tris (pH 7.3), 1 mM EGTA and 1 mM DTT. The suspension was centrifuged at 140,000 x g for 40 min. The resulting pellets were suspended in a solubilizing buffer containing 10 mM MES-Tris (pH 7.3), 0.25 M mannitol, 1 mM EGTA, 1mM DTT, and 20% glycerol(w/v) and stored at -80°C for up to 1 week.

Solubilization of tonoplast H^+-ATPase

Tonoplast ATPase was solubilized from the 0.1 % deoxycholate (DOC) pellet using 30mM octyl-d-glucoside (OG) as described by Mandala and Taiz[29].

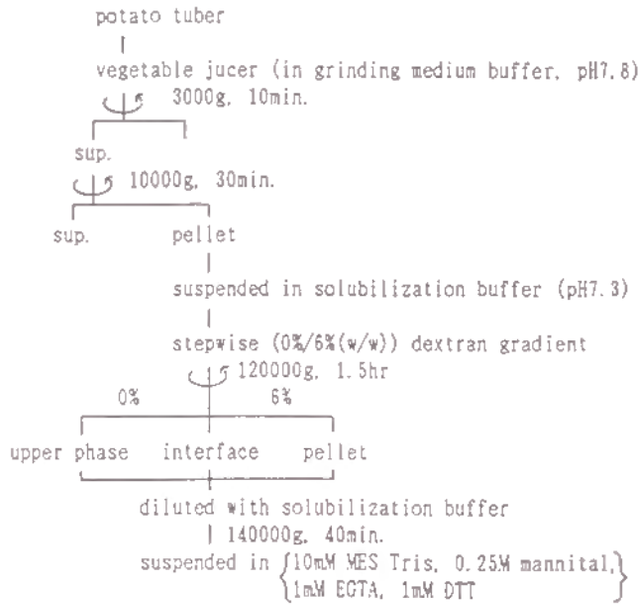


Fig. IV - 4 Isolation of Tonoplast-enriched Membrane.

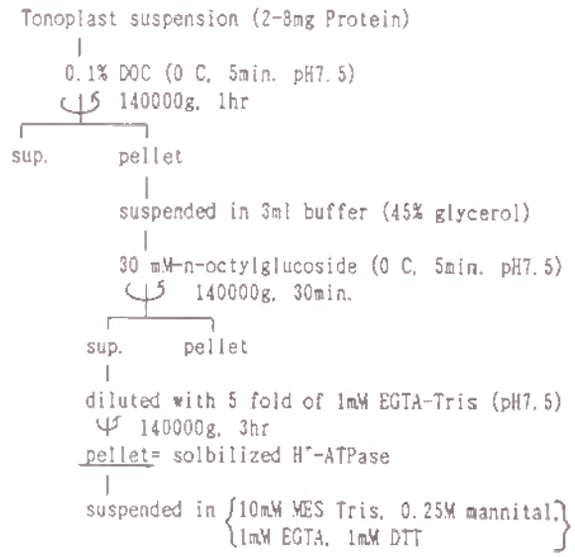


Fig. IV - 5 Solubilization of Tonoplast H+-ATPase

The details were shown in Fig. IV–5. The solubilized fraction from tonoplast membrane was diluted by adding 5 volume of 1mM EGTA–Tris (pH 7.5) and centrifuged at 140,000 x g for 3h. The final pellet was suspended at a protein concentration about 3mg/ml in the solubilizing buffer.

Irradiation of potato tubers(in vivo irradiation)

Potato tubers were irradiated at 0.1 to 1 kGy with a Gamma–cell 220(AECL Canada, 6.2kGy/h) in ambient atmosphere. After irradiation potatoes were stored at 5 °C in a dark room.

Irradiation of tonoplast (in vitro–irradiation)

The suspensions of tonoplast obtained from unirradiated potatoes were divided into 4 aliquots in eppendorf tubes and protein concentration was 7.4mg/ml, and irradiated at doses of 0, 0.1, 0.5 and 1.0 kGy on ice. At standing for 2h and 12h on ice after the irradiation, the activity of ATPase and the rate of ATP–dependent H^+–pumping, FFA contents were determined from irradiated membranes.

Irradiation of solubilzied H^+–ATPase (enzyme–irradiation)

Solutions of solubilized H^+–ATPase in solubilizing buffer were irradiated on ice, and determined ATPase activity after 2 and 12h irradiation.

Assay for ATPase activity and protein determination

ATPase activity was measured at 38°C with 20–30μg of protein per assay for membrane vesicles and 10μg for solubilized enzyme [30]. The reaction was initiated by the addition of membrane or solubilizied ATPase to a 0.5 ml reaction mixture containing 3mM Tris–ATP (Boehringar), 3mM $MgSO_4$, 50mM KCl, 1 mM ammonium molybdate, 2mM azide, 50μM vanadate and 30 mM MES–Tris (pH 7.5), and terminated after 30 min by addition of 100 μl of 50% TCA. Phosphate released from ATP was determined according the method of Kasamo et al.[31].

Proteins were quantitated by the method of Lowry et al., as modified by Peterson[32], using bovine serum albumin as the standard.

Measurement of H^+–pumping

H^+–pumping was measured by monitoring the fluorescence quenching with quinacrine [17]. Vacuolar membranes vesicles prepared from irradiated tubers and irradiated vacuolar membrane vesicles were incubated in 2.0ml of reaction medium

containing 0.25M sucrose, 100mM KCl, 10mM Mes–Tris (pH 6.5) and 2 μM quinacrine at room temperature. The reaction was initiated by the addition of 3mM Mg–ATP. H^+–pumping was measured by monitoring the decrease in quinacrine fluorescence with a spectrofluorometer (model RF–5000; Shimadzu, Kyoto, Japan). H^+–pumping was expressed as the initial rate of fluorescence quenching.

Extraction and analysis of lipids

Lipids were extracted from the vacuolar membrane by the method of Bligh and Dyer[33]. In order to avoid deterioration of membrane lipid, 0.05% of BHT was added to the extraction solvents. Fatty acid compositions of total lipid were determined by the analysis of methyl esters with a gas–chromathograph according to the method previously described (Chapter III). Free fatty acids and lipid hydroperoxide content in the lipid fraction were determined by the methods previously described (Chapter IV)

ATPase assay in the presence of lipids

The effects of lipids extracted from irradiated tonoplast on ATPase were studied by measuring the activity of ATPase in the presence of the lipids as follows, 9.4 μg of tonoplast lipid in chloroform solution was taken in a eppendorf tube, and the solvent was removed by nitrogen gas. The dried lipid was then hydrated with a 30μl of ATPase solution containing 14.9 μg protein, 10 mM MES–Tris (pH 7.3), 0.25 M mannitol, 1 mM EGTA, 1mM DTT, and 20% glycerol (w/v), and mixed the sample with a Vortex mixer for 30 s, followed by ultrasonic irradiation in a sonifier (IUC–2811, Tochu, Japan) for 1 min. After standing at for 20 min 25 °C, the mixture was added to a 0.5 ml of reaction mixture to ATPase activity as mentioned above.

Results

Preparation of tonoplast ATPase from potato tubers

Tonoplast enriched fractions were obtained from a 3,000 to 10,000 x g pellet by the dextrane T–70 step gradient. ATPase activity was localized in this fraction, and 81 % of the activity was inhibited with 100mM KNO_3. The specific activity of tonoplast membrane from unirradiated potato tubers was 0.136 ± 0.012 μmole Pi mg protein^{-1} min^{-1}(n = 16). The ATPase was insensitive to vanadate, inhibitor of plasma membrane ATPase, azide, inhibitor of mitochondrial ATPase, and ammonium molybdate, inhibitor for non–specific phosphatase.

ATPase bounded to tonoplast membrane was solubilized by a two step procedure using DOC and OG. The specific activity of solubilized enzyme was 0.221±0.006 μmole Pi mg protein^{-1} min^{-1}(n = 4).

Inactivation of tonoplast H^+–ATPase by gamma–irradiation of potato tube

Vacuolar membrane vesicles from unirradiated potato tubers were equilibrated with quinacrine for 3 min to obtain a stable base line. When MgATP was added, the quenching of quinacrine fluorescence was started and it reached maximum value after about 12 min. In the case of the tonoplast vesicles from irradiated tuber 12h after irradiation. The time required to obtain stable baseline was slightly longer, compared with that of unirradiated one, it took about 5 min (Fig. IV–6).

The initial rate of proton–pumping across the vacuolar membrane from the tuber 2h after irradiation at 0.1 kGy reduced significantly, although ATPase activity in the same membrane was not significantly reduced. At the other doses it was restored in tonoplast from the tubers 2h after irradiation. H^+–pumping was strongly suppressed 12h after irradiation at any dose. It reduced to 40 % of unirradiated tuber at 1kGy (Fig. IV– 7a).

Figure IV–7b shows the effect of gamma–irradiation of potato tuber on tonoplast ATPase activity and H^+–pumping. The ATPase activities were slightly lower in the tonoplast from the irradiated tubers 2h after irradiation at any dose as compared with that of unirradiated tubers. The ATPase activities were markedly decreased in tonoplast membrane from irradiated tubers 12h after irradiation. The relative activity in irradiated tubers (1kGy) were 60% of unirradiated tuber. There was no clear dependence of ATPase inactivation on the radiation dose.

Table IV–1 shows the ATPase activity and H^+–pumping in vacuolar membrane during the storage of potato tubers irradiated at 1.0 kGy. The inactivation of ATPase activity and H^+–pumping by irradiation of potato tuber was maximally noted at 12h after irradiation, after that it decreased with in crease in the time. The ATPase activity was observed at the same level of unirradiated tuber 2 days after irradiation.

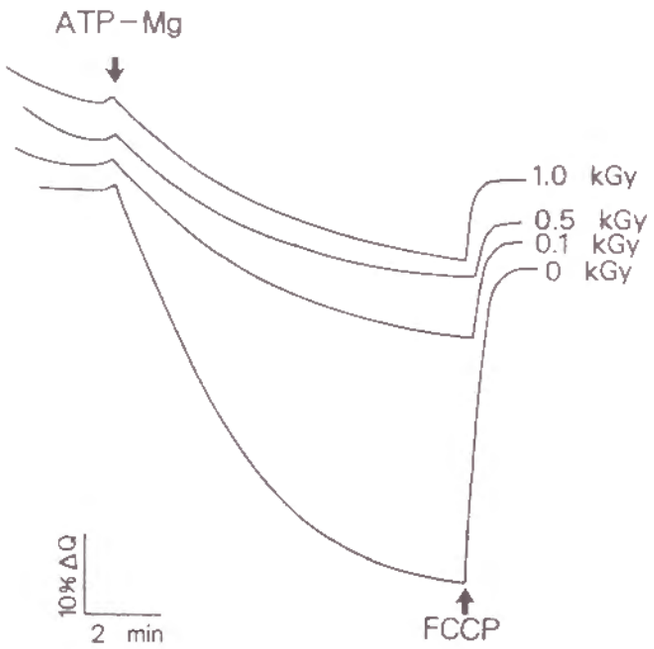


Fig. IV - 6 Quencing(Q) of Quinacrine Flouresscence by Tonoplast Vesicles Prepared from Gamma-irradiation Potato Tubers 12h after Irradiation.

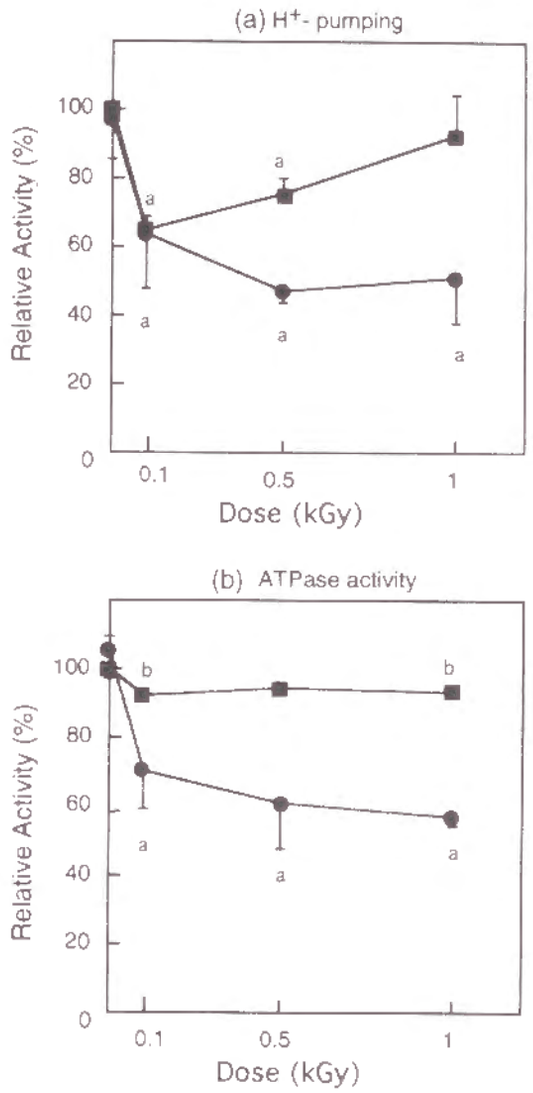
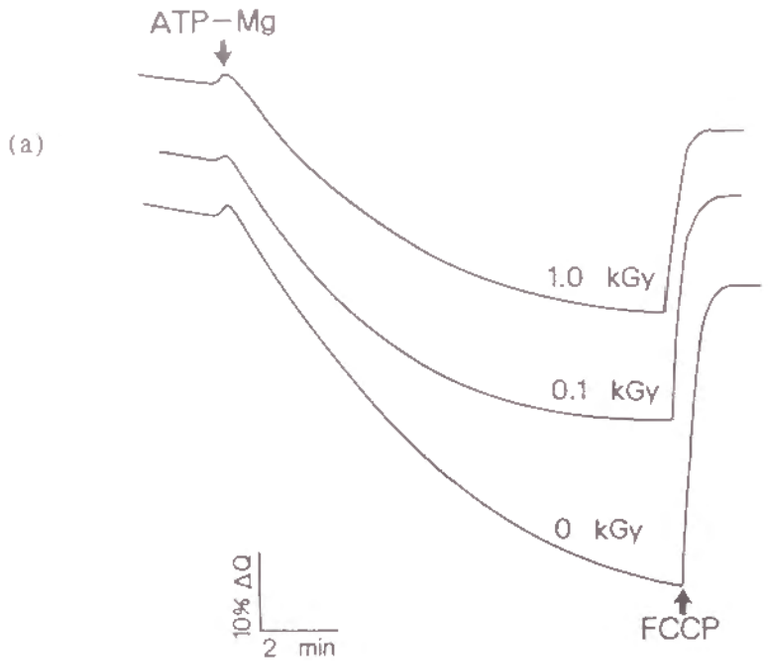


Fig. IV- 7 Effect of Gamma-irradiation of Potato Tuber (in vivo) on the Tonoplast H^+-pumping (a) and H^+-ATPase activity.

Tonoplast vesicles were prepared from potato tubers at 2h (■) and 12h (●) after irradiation.

ATPase activity and H^+-pumping were expressed as relative values (%) of control (0 kGy 2h) (mean values + S.D. (n=3)).

Control ATPase activity was 0.135 + 0.006 μmole Pi (mg protein)$^{-1}$ min^{-1}.
Statistically significant difference was evaluted against unirradiated potato tuber at 2h (control); a, $P < 0.01$, b, $P < 0.05$.



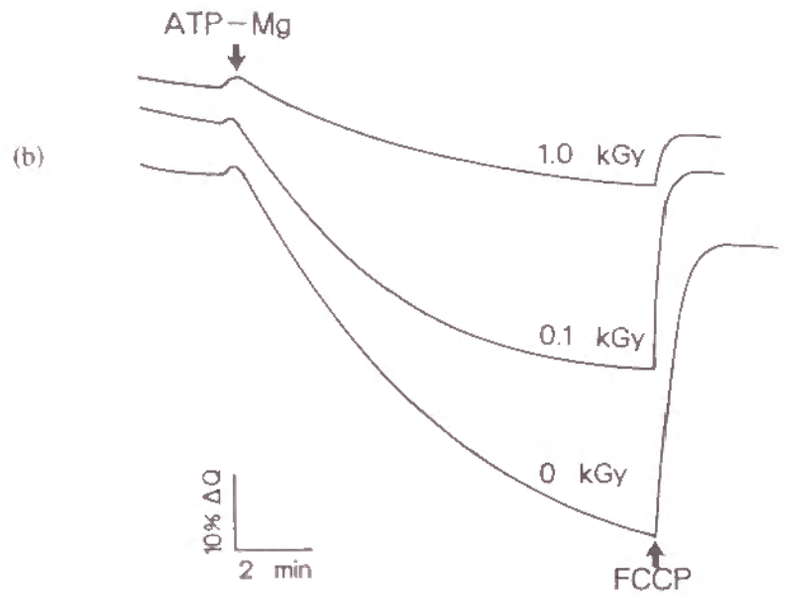
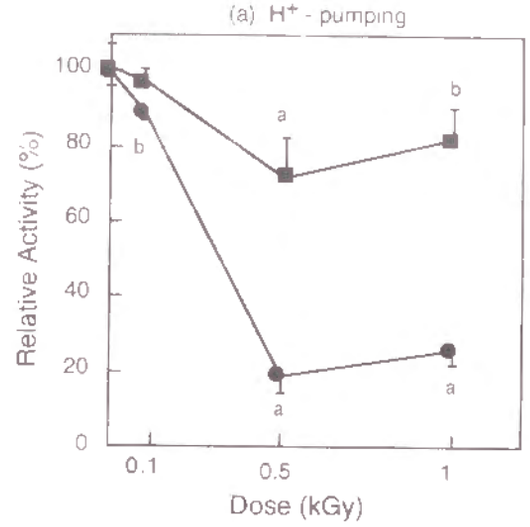


Fig. IV - 8 Quenching (Q) of Quinocrine Fruoresence by Vacuolar Membrane Vesicles 2h (a) and 12h (b) after irradiation



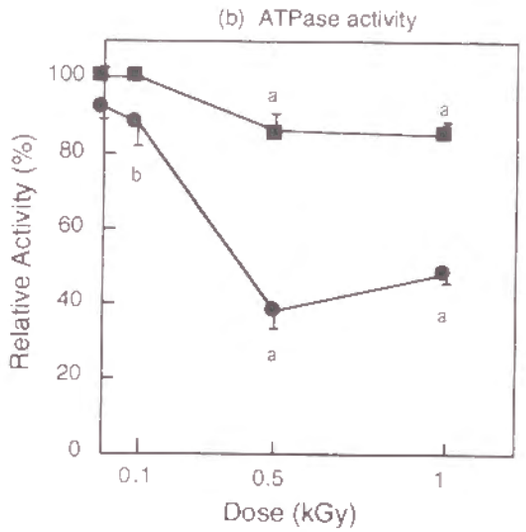


Fig. IV- 9 H^+-pumping (a) and ATPase (b) Activities in Gamma-irradiated Vacuolar membrane Versicles from Potato Tuber.

Tonoplast vesicles from potato tubers were suspended in 10mM MESTris buffer (ph 7.3) containing 0.25M mannitol, 1 mM EGTA, 1 mM DTT and 20% (W/V) grycerol and irradiated in ambient atmosphere.

ATPase activity and H^+- pumping were determined after incubation of membrane suspensions for 2h (■) and 12h (●) at 0°C.

ATPase and H^+- pumping activities were expressed as relative values (%) of control (0 kGy 2h).

Control ATPase activitiy was 0.134 + 0.004 μmole Pi (mg protein)$^{-1}$ min^{-1}.
Statistically siginigicant difference was evaluted against control: a, $P< 0.01$, b, $P < 0.05$.

Table IV–1. ATPase activity and H^+–pumping in the tonoplast vesicles from gamma–irradiated potato tuber during the storage at 5 °C.

(mean values + S.D.(n=3))

Dose (storage period)	ATPase activity* (%)	H^+–pumping* (%)
0 kGy (2h)	100 ± 4.3	100 ± 20.9
0 kGy (12h)	105 ± 8.1	96 ± 11.2
0 kGy (10d)	95 ± 3.7	94 ± 11.0
1.0 kGy (2h)	93 ± 1.2[b]	95 ±12.1
1.0 kGy (12h)	60 ± 11.1[a]	44 ±3.9[a]
1.0 kGy (1d)	94 ± 4.8	70 ± 4.2[b]
1.0 kGy (2d)	97 ± 2.5	86 ± 10.8
1.0 kGy (3d)	95 ± 3.5	87 ± 12.3
1.0 kGy (4d)	100 ± 4.4	83 ± 3.9
1.0 kGy (6d)	98 ± 3.5	87 ± 4.9
1.0 kGy (10d)	88 ± 2.9	92 ± 3.0

*Relative activity (%) of control (0 kGy (2h)).
Control activity of tonoplast H^+–ATPase was 0.135 ± 0.002
Statistically significant difference was evaluated against control;
a, $P < 0.01$, b, $P<0.05$

Inactivation of tonoplast H^+–ATPase by gamma–irradiation of isolated vacuolar membrane from potato tuber

Figure IV–8 shows the example of quenching of quinacrine fluorescence by gamma–irradiated vacuolar membrane vesicles. The initial rate of fluorescence quenching decreased with increase in radiation dose and postirrdiation period. Figure IV–9 shows the ATPase activity and H^+–pumping in irradiated vacuolar membrane. Dose dependent inactivation were clearly observed up to 0.5 kGy. The activities decreased markedly 12h after irradiation at 0°C.

Inactivation of solubilized ATPase by gamma–irradiation

When ATPase solubilized from potato vacuolar membrane was irradiated in the buffer solution (enzyme–irradiation), the ATPase was drastically inactivated depending on the doses at 2h after irradiation. The activity was reduced to about 50% of unirradiated enzyme by the irradiation at 1 kGy. When the irradiated samples were kept on ice for 12h, the activities still remained almost at the same levels as those at 2h after irradiation (Fig. IV–10).

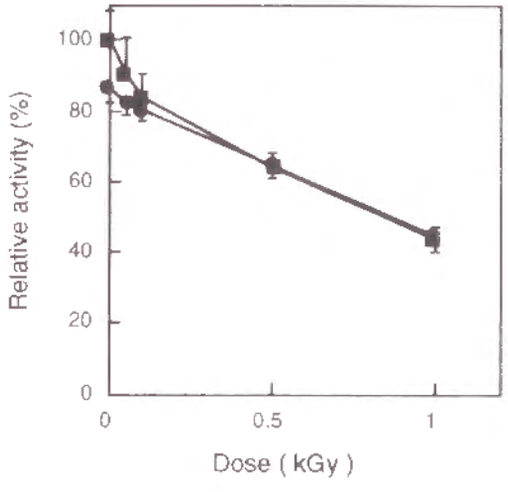


Fig. IV - 10 Effect of Gamma-irradiation on the Activities of Solubilized Tonoplast H^+-ATPase from Potato Tuber.

Solubilized H^+-ATPase were suspended in 10 mM Mes-tris buffer (pH. 7.3) containing 0.25M mannitol, 1 mM EGTA, 1 mMDTT and 20% (W/V) grycerol and irradiated with gamma-rays. ATPase were assayed after incubation for 2h (■) and 12h (●) at 0°C.
Control ATPase activity was 0.221 ± 0.006.
ATPase activity was expressed as relative values (%) of control (0 kGy 2h).
Statistically significant difference was evaluated against unirradiated ATPase (control).

Effect of gamma-irradiation on vacuolar membrane lipids

Lipids were extracted from irradiated tonoplast (*in vitro*) and tonoplast of irradiated tubers (*in vivo*).

Table IV-2.

FFA contents of vacuolar membranes from potato tuber irradiated with gamma-rays in vivo. (Mean values + S.D. (n = 3))

Dose* (post-irradiation period)	Total FFA (nmole/mg protein)	FFA composition (mol %)				
		16:0	18:0	18:1	18:2	18:3
0 kGy (2h)	91.4 ±3.5	50.1	5.8	5.4	22.9	5.8
0.1 kGy (2h)	129.5 ±7.2[b]	46.4	9.4	2.4	33.8	8.1
0.5 kGy (2h)	143.1 ±21.0[a]	38.4	10.5	3.2	38.8	8.9
1.0 kGy (2h)	125.5 ±7.2[a]	44.9	12.4	4.9	29.6	8.1
0 kGy (12h)	98.7 ±14.1	46.9	12.8	3.0	29.6	7.0
0.1 kGy(12h)	139.9 ±6.5[a]	39.9	12.0	3.3	35.1	9.5
0.5 kGy(12h)	224.8 ±26.2[a]	37.1	9.5	1.4	43.1	8.9
1.0 kGy(12h)	136.6 ±15.9[a]	42.8	12.5	2.3	34.5	7.8

*Lipids were extracted from the vacuolar membrane vesicles prepared 2h and 12h after irradiation of potato tubers.
Statistically significant difference was evaluated against control (0 kGy (2h)); a, $P < 0.01$, b, $P<0.05$.

Table IV-3.

FFA contents of vacuolar membranes irradiated with gamma-rays in vitro. (Mean values + S.D. (n = 3))

Dose* (post-irradiation period)	Total FFA (nmole/mg protein)	FFA composition (mol %)				
		16:0	18:0	18:1	18:2	18:3
0 kGy (2h)	90.7 ±2.6	44.8	10.8	3.6	31.4	9.4
0.1 kGy (2h)	101.0 ±11.3[b]	46.4	9.4	2.4	33.8	8.1
0.5 kGy (2h)	114.0 ±7.9[a]	41.9	9.8	2.3	36.8	9.2
1.0 kGy (2h)	114.4 ±4.0[a]	41.9	10.6	2.1	36.8	8.6
0 kGy (12h)	96.4 ± 1.5	46.1	10.3	2.3	33.5	8.0
0.1 kGy(12h)	104.4 ±6.9[a]	44.8	10.2	2.5	34.3	8.1
0.5 kGy(12h)	140.6 ±11.7[a]	40.4	8.2	2.0	41.3	8.9
1.0 kGy(12h)	159.0 ±6.6[a]	38.8	7.9	2.3	40.3	10.6

*Lipids were extracted from the vacuolar membrane vesicles 2h and 12h after irradiation.
Statistically significant difference was evaluated against control (0 kGy (2h)); a, $P < 0.01$, b, $P<0.05$.

Either type of irradiation, in vivo or in vitro irradiation, did not bring about the accumulation of lipid hydroperoxide in the vacuolar membrane. However, free fatty acid (FFA) contents were significantly higher in the vacuolar membrane from irradiated tuber as compared with that of unirradiated tuber. The proportion of poly-unsaturated free fatty acid (18:2 and 18:3) increased with the increase in total FFA (Table IV-2).

The accumulation of FFA was also observed in irradiated vacuolar membranes depending on the radiation dose. The FFA content increased during the postirradiation period (table IV- 3).

Effect of total lipid extracted from gamma-irradiated tonoplast on the activity of solubilized ATPase

Table IV-4.
Effect of lipids extracted from gamma-irradiated vacuolar membrane on the activity of solubilized H^+-ATPase.

Extracted lipid* Dose (hours after irradiation)	ATPase activity $\mu Pi(mg\ protein)^{-1}min^{-1}$
None	0.203 ± 0.018**
unirradiated	0.162 ± 0.040
1.0 kGy (2h)	0.087 ± 0.006[a]
1.0 kGy (12h)	0.046 ± 0.017[ab]

* Total lipids were extracted from unirradiated vacuolar membrane vesicles, membrane irradiated at 1 kGy and stored for 2h(1kGy (2h)), and 12h(1kGy(12h))at 0°C.

Lipid(9.4μg) and solubilized ATPase (14.9 μg protein) were mixed in 30 μl of buffer solution for 20min at 25°C.

Enzyme reaction was initiated by the addition of lipid-protein mixture into 0.5ml of reaction mixture. Enzyme reaction was carried out in the same manor as describe above.

**Mean values + S.D. (n=4).
Statistically significant difference evaluated against 0 kGy(2h); a, and 1kGy (2h);b, respectively ($p< 0.01$).

The lipids were extracted from irradiated tonoplast and solubilized ATPase activity was measured in the presence of the extracted lipids (Table IV–4). ATPase activity was inhibited by the exogenously adding of the lipids extracted from vacuolar membranes. The inhibitory effect of the lipids from irradiated tonoplast (1kGy) was larger than that of lipids from unirradiated one. The lipids extracted from irradiated tonoplast 12h after irradiation inhibited the enzyme activity to a grater extent as compared with that of 2h after irradiation at the same dose (1 kGy).

Discussion

The H^+–translocating ATPase in tonoplast of potato tubers was severely inactivated by gamma–irradiation as well as other type of ATPase in various origins, such as Na^+–ATPase in porcine erythrocyte membrane [34] and mitochondrial membrane ATPase from germ seedling[35]. As shown in the present study, the extent of inactivation of tonoplast H^+–ATPase and H^+–pumping after irradiation varied considerably with radiation conditions (*in vivo*–irradiation, *in vitro*–irradiation and enzyme–irradiation) (Fig. IV 7, 9,10).

The activity of solubilized H^+–ATPase was markedly reduced by enzyme–irradiation with dose dependency up to 1 kGy (Fig. IV–10). The reduction of H^+–ATPase activity was not proceed during the period after irradiation (Fig. IV–10). This indicates that the radiation may attack directly H^+–ATPase molecules. Yamanishi and Kasamo [36] demonstrated the essential role of cysteine residues near the catalytic site of tonoplast H^+–ATPase from mung bean hypocotyls. Sulfhydryl(SH) groups of membrane protein is known to be sensitive target of irradiation[37),38)]. Solubilized tonoplast H^+–ATPase might be sensitive to ionizing radiation and be inactivated directly by the reaction such as oxidation of SH groups.

The rate of inactivation of H^+–ATPase and H^+–pumping 2h after *in vivo*– and *in vitro*–irradiation was enhanced during 12h after irradiation (Fig.IV–7,9 and Table IV–1). These mean that further inactivation after *in vivo*– and *in vitro*–irradiations may proceed through metabolic functions of membranes. Free radical (FR) has a hydrophilic nature, which is unfavorable their interaction with lipids in hydrophobic

core of membranes[39] and lipid soluble anti–oxidants e.g. vitamin E exist in the membrane system [40]. When these points are taken into consideration, the direct effect of FR on the ATPase exists in lipid bilayer may be eliminated. It is well known that the activities of membrane–bound enzyme depends to a great extent on the lipid constituent of biological membrane[1]. Lipid modulation of tonoplast H^+–ATPase from mung bean[41] has been reported as well as plasma membrane H^+–ATPase[42),43)]. The inhibition caused by FFA, especially unsaturated FFA, was also demonstrated in purified H^+–ATPase of plasma membrane from rice [43], purified H^+–ATPase of tonoplast membrane from mung bean hypocotyls [41] and Na,K–ATPase in rabbit kidney [44]. Either *in vivo*–irradiation and *in vitro*–irradiations did not brought about the accumulation of lipid hydroperoxide in tonoplast membrane (data not shown). The de–esterification of membrane phospholipid was reported to be stimulated directly by FR[45),46)],. Voisine et al.[25] reported that gamma–irradiation of cauliflower bud proceeded the deterioration of microsomal membrane lipid accompanied by FFA accumulation. In these cases the increase in lipid hydroperoxide was not obvious. In the present study, dose dependency of FFA accumulation was shown in vacuolar membranes 12h after *in vitro*–irradiation (Table IV–3), which were concomitant with the strong inhibition of the ATPase activity and H^+–pumping (Fig. IV–9). FFA contents in vacuolar membranes of potato tubers were increased by *in vivo*–irradiation with increase in dose up to 0.5 kGy (Table IV–2). FFA contents in vacuolar membranes 12h after *in vivo*– and *in vitro* irradiation is much higher than that 2h after their irradiations (Table IV–2 ,3). The strong inactivation of H^+–ATPase activity 12h after irradiation would be partly attributable to the accumulation of FFA in vacuolar membrane.

Table IV–4 clearly demonstrated that the lipids from irradiated tonoplast vesicles, which contain large amount of FFA, could strongly inhibit the activity of H^+–ATPase solubilized from unirradiated vacuolar membrane. The ratio of total FFA to enzyme protein for ATPase assay was 76.5 nmole FFA/mg protein for unirradiated control (0kGy 2h), and those for tonoplast 2h and 12h after irradiation at 1 kGy were 87.9 nmole/mg protein and 112 nmole/mg protein, respectively. These values were almost the same level as in intact vacuolar membranes. In unirradiated control the concentra–

tion of 18:3 and 18:2 containing in the reaction mixture were estimated at 3.5μM and 11.9μM, respectively. For tonoplast vesicles 2h after irradiation at 1 kGy the concentrations of 18:3 and 18:2 were 3.8μM and 16.3μM, respectively. And in case of 12h after irradiation at 1kGy they were 5.9μM and 22.4μM, respectively. The effect of FFA on various type of ATPase still remains obscure. Palmgren et al.[47] reported that narrow range of free fatty acids stimulated the plasma membrane H^+-ATPase from oat root. Macri et al.[48] reported that unsaturated FFA slightly stimulated the ATPase activity in pea stem microsomes. Im and Blakeman[49] showed unsaturated fatty acids 18:2 and 18:3 ca. 10^{-5}M markedly inhibited H^+-K^+ATPase in hog and rat gastric membranes. Yamanishi and Kasamo[41] reported that purified tonoplast H^+-ATPase from mung bean hypocotyl was strongly inhibited by exogenous addition of 18:2 and 18:3, but not by saturated fatty acid. Swarts et al.[44] extensively studied the inhibitory mechanisms of purified Na,K-ATPase from Rabbit kidney by FFA. They revealed that ATPase was inhibited by FFA through two different effect, the effect on the dephosphorylation (short term effect) and irreversible inactivation (long term effect), which was strongly dependent on reaction conditions; temperature, enzyme concentration, time and fatty acid concentration. Although further investigations are need to clarify the mechanism of regulation of ATPase activity by FFA, in the present study, the inhibitory effect of 10^{-5}M level of FFA on solubilized tonoplast H^+-ATPase was observed by the pre-incubation of enzyme with lipids for 20 min(Table 4). We did not show the inhibitory effect on H^+-pumping across vacuolar membranes by means of exogenous FFA, however Macri et al.[48] reported that FFA as linoleic acid and linolenic acid drastically inhibited ATP-dependent H^+-pumping in pea stem microsomes.

The decrease in H^+-pumping across vacuolar membrane vesicles by *in vivo*-irradiation (Fig.IV-7) and *in vitro*-irradiation (Fig.IV-9) was larger extent than that of ATPase activity. Thus H^+-pumping might be more sensitive to gamma-irradiation than ATPase activity. Sarafin et al. [50] reported, in the estimation of the molecular mass by radiation inactivation, that the H^+ transport was more sensitive to gamma-irradiation than ATP hydrolysis in tonoplast H^+-ATPase from red beet, which is consistent with present observation for potato tonoplast H^+-ATPase.

The ATPase activity and H^+-pumping once declined with irradiation were recovered to the control level within 2 days after irradiation(Table IV- 1). As previously described the membrane permeability of irradiated potato tubers increased immediately after irradiation and then continued to increase for up to 2 days, followed by a gradual decrease. Mckersie et al. [15] showed that FFA accumulation accelerates membrane permeability in model system, which suggested increase in electrolyte leakage caused by irradiation was attributable to the increase in FFA content. The recovery of ATPase activity (Table VI-1) and membrane permeability to the starting level during 2 days and a few weeks after irradiation strongly shows the return to normal function of the membranes during this period, which were concomitant with the increase in membrane phospholipids.

In conclusion, tonoplast H^+-ATPase and H^+-pumping across vacuolar membranes were reduced by *in vivo*- and *in vitro*-irradiations. The radiation-induced inactivation of H^+-ATPase in potato tubers was amplified by the deterioration of membrane lipids caused with gamma-irradiation, especially accumulation of FFA in addition to the direct effect on enzyme molecules.

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Summary

Chapter I

The doses of gamma-rays and electron beams were evaluated by Fricke dosimetry. The responses of cellulose triacetate dosimeter (CTA) and radiochromic film dosimeter (RCF) to the two types of radiations were compared to investigate their dose rate dependence. Both the change in absorbance at 280nm of CTA and that in absorbance at 510nm of RCF caused by gamma-irradiation were larger than those by electron-irradiation, when the dosimeters were irradiated to the same dose, which suggests that the responses of CTA and RCF are dependent upon dose rate. Based on these results, the dosimetry method of gamma-rays and electron beams with film dosimeters was established for the experiments in the present study.

Chapter II

Phosphatidylcholine was irradiated in the state of film or liposome with gamma-rays or electron beams, and the amount of peroxide was determined to compare the effects of the two types of radiation. The amount of peroxide formed in both film and liposome with gamma-rays was significantly larger than that with electron beams, when the samples were irradiated at the same dose. Proteins such as bacteriorhodopsin lowered the degree of peroxide formation in liposome, and the effect of gamma-rays was much larger than that of electron beams, even in the presence of protein. The results in the present investigation indicate that the effects of gamma-rays on peroxide formation of phosphatidylcholine were significantly larger than those of electron beams irrespective of the state of the lipid.

Chapter III

The lipid composition of irradiated potato (Solanum tuberosum) tubers was determined to investigate the effect of gamma-irradiation on potato tuber membranes. Neither accumulation of lipid hydroperoxide nor significant loss of membrane lipid were obvious in gamma-irradiated potato tuber within a day after irradiation. Free fatty acid content was slightly higher in the tubers 12h after irradiation as compared with unirradiated ones. Phospholipid content of potato tubers increased for a few weeks after irradiation Linolenic acid contents of glycolipids, especially monogalactosyl diacylglycerol and digalactosyl diacylglycerol, significantly increased, accompanied by a decrease in the linoleic acid contents, for a few weeks after irradiation. These changes were shown in all of subcellular fractions or regions of potato tubers. Fatty acid synthesis and desaturation induced by mechanical injury was retarded by prior irradiation of potato tuber.

Chapter IV

The physicochemical properties of membranes and activity of tonoplast H^+-ATPase were investigated in irradiated potato tubers in accordance with the compositional changes of membrane lipids. The impedance parameter altered to a greater extent in the region of potato tuber which showed the larger increase in membrane lipid contents by gamma-irradiation.

The rate of the electrolyte leakage from potato disks increased within 1 hour after irradiation (1kGy) and continued to increase for a few days, followed by a decrease to the level of unirradiated ones. The restoration of membrane permeability was observed concomitant with the increase in membrane lipids.

Tonoplast H^+-ATPase and H^+-pumping across vacuolar membranes were significantly reduced by the gamma-irradiation of potato tuber 12h after irradiation. The amount of free fatty acid in vacuolar membrane increased during post-irradiation period with the decrease in the H^+-ATPase activity. The activity of H^+-ATPase solubilized from the vacuolar membrane of unirradiated potato tubers was strongly inhibited by lipids of irradiated tonoplast vesicles. These results indicated that the radiation induced inactivation of H^+-ATPase in potato tubers was amplified by the degradation of membrane lipid caused by gamma-irradiation, especially accumulation of free fatty acid, in addition to the direct effect on enzyme molecules.

The ATPase activity reduced by gamma-irradiation was restored to the same levels of unirradiated tubers during the storage, which were also concomitant with the increase in membrane phospholipid.

List of Abbreviations

CTA; cellulose triacetate film dosimeter

RCF; radiochromic film dosimeter

PC; phosphatidylcholine

FA; fatty acid

TFA; esterified and unesterified fatty acid

FFA; unesterified free fatty acid

16:0; palmitic acid

18:0; stearic acid

18:1; oleic acid

18:2; linoleic acid

18:3; linolenic acid

NL; neutral lipids

PL; phospholipids

GL; glycolipids

MGDG; monogalactosyl diacylglycerol

DGDG; digalactosyl diacylglycerol

Pm; plasmalemma

Tp; tonoplast

Mt; mitochondoria

Er; endoplasmic reticulum

Am; amyloplast

Mops; 3-(N-Morpholino)propanesulfonic acid

EDTA ;ethylenediaminetetraacetic acid

EGTA; ethyleneglycol-bis(β-aminoethyl) ether

DTT; dithiothreitol

Mes; 2-(N-morpholino)-ethanesulfonic acid

Tris; tris(hydroxymethyl)aminomethane

DOC; sodium deoxy cholate

OG; octyl-β-glucopyranoside

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